Organotypische Slicekulturen von humanem *Glioblastoma multiforme* als Testsystem für neue Therapien

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Referat:

Glioblastoma multiforme (GBM) ist der nach WHO am gefährlichsten eingestufte Hirntumor astrozytären Ursprungs. Patienten versterben ohne Behandlung etwa drei bis sechs Monaten nach Diagnose, die derzeitig modernste Behandlung mit Chemo-Radiotherapie verlängert das mediane Überleben auf 12-15 Monate. Trotz intensiver Forschung gibt es zurzeit keine realistische Heilungschance. Bislang erfolgt der Großteil der Forschung an Zellkulturen oder mit Hilfe von Tiermodellen, bei denen ein Tumor künstlich erzeugt wird. Dabei ergeben sich Probleme für die Übertragung der Ergebnisse auf den Menschen. Zellkulturen werden z.B. als sogenannte Monolayer-Kulturen gehalten, was bedeutet, dass ihnen der natürliche Gewebeverband und die für Signalling-Wege wichtige extrazelluläre Matrix fehlen. Außerdem werden solche Langzeitkulturen häufig subkultiviert und mutieren dadurch in Richtung einer klonalen Linie, was zwar Ergebnisse leichter reproduzierbar macht, aber nicht die Situation im Patienten widerspiegelt. Tierversuche implizieren zwar den Gewebeverband im Körper, jedoch müssen die dafür verwendeten Nager immunsupprimiert sein, so dass sie den induzierten Tumor nicht abstoßen. Dies erzeugt wiederum ein künstliches Umfeld.

In diesem Projekt wird untersucht, ob sich humane GBM-Gewebe als sogenannte Slice-Kultur halten lassen und als Testsysteme zur Untersuchung der Wirkung von Chemotherapeutika sowie Bestrahlung geeignet sind. Bei dieser Kultivierungsmethode wird das Gewebe in Scheiben (Slices) geschnitten, wobei alle Zellen im Verband sowie die 3D-Struktur erhalten bleiben. Wegen des humanen Ursprungs entfällt das Problem des Speziesunterschiedes. Das Gewebe wird direkt aus dem Operationssaal ins Labor transferiert und weiterverarbeitet. Wir konnten bislang zeigen, dass Slice-Kulturen von humanem GBM über mindestens zwei Wochen in Kultur vital bleiben und ihre ursprüngliche charakteristische Morphologie beibehalten. Etablierte Behandlungsmethoden wie die Gabe von Temozolomid oder Röntgenbestrahlung zeigen auch in kultivierten Slices bekannte Effekte wie Induktion von DNA-Doppelstrangbrüchen, Reduktion von Proliferation und Aktivierung des Apoptose-Enzyms Caspase 3. Eine neue Therapieoption besteht seit einigen Jahren in der Bestrahlung mit Kohlenstoffionen (^{12}C), die an der GSI Helmholtzzentrum für Schwerionenforschung in Darmstadt entwickelt und getestet wurde. Derzeit wird diese Therapie sehr erfolgreich an soliden Tumoren im Kopf- und Halsbereich angewendet und soll nun auf weitere Tumorarten ausgedehnt werden. Eine Kooperation mit der dortigen Biophysik-Gruppe wurde initiiert, um humane GBM-Slices mit ^{12}C zu bestrahlen. Bislang wurde das entsprechende Setup etabliert und erste Experimente durchgeführt. Die ersten Ergebnisse wurden kürzlich publiziert. Weiterhin soll nun geprüft werden, ob das Ansprechen der GBM Slice-Kulturen mit dem Überleben der Patienten korreliert bzw. ob resistente Kulturen aus Patienten stammten, die schlecht auf die Therapie reagierten. Außerdem sollen überlebende Zellen in den Slices nach Behandlung auf ihre molekularen Eigenschaften geprüft werden, um Hinweise auf die Mechanismen der Tumorresistenz zu erhalten. Langfristig könnten diese Slice-Kulturen genutzt werden, um neuartige Wirkstoffe in der Vorklinik zu prüfen oder eine optimierte, personalisierte Therapie für Patienten zu ermitteln.

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1. Einleitung

1.1 *Glioblastoma multiforme*

Glioblastoma multiforme (GBM) ist der aggressivste Hirntumor astrozytären Ursprungs und wird von der Weltgesundheitsorganisation (*World Health Organisation,* WHO) unter den astroglialen Tumoren als der gefährlichste eingestuft (Grade IV; Louis et al., 2007). Es kann als Primärtumor auftreten (*de novo*) oder sich aus einem niedergradigen Astrozytom entwickeln (sekundäres GBM). Die Kriterien zur Diagnose eines GBM sind hohe Zelldichte, pleomorphe Zellkerne, Proliferation bzw. Mitosefiguren, Palisadenstruktur, Neubildung von Blutgefäßen sowie deren Invasion und die Entstehung nekrotischer Bereiche (Abb. 1) (Böcker et al., 2008; Louis DN, 2006; Altman et al., 2007). Unter den Tumoren des Zentralen Nervensystems hat das GBM mit 33,1% in Europa einen großen Anteil. Die 5-Jahres-Überlebensrate beträgt trotz modernster radio-chemotherapeutischer Behandlung lediglich 2,7%, während Patienten ohne Behandlung meist innerhalb weniger Monate versterben (Sant et al., 2012).

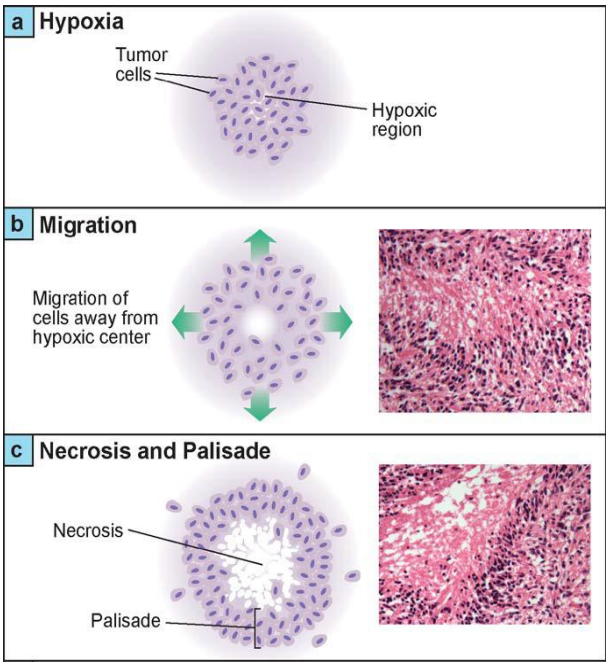

Abb. 1: Charakteristische Eigenschaften von *Glioblastoma multiforme*. Wegen der hohen Proliferation im Tumorinneren entstehen hypoxische Bereiche (a), aus denen die Zellen migrieren (b). Dadurch entstehen zentrale Nekrosen mit palisadenartigen Strukturen in den Randbereichen (c). (modifiziert nach Louis DN, 2006).

Über mögliche Ursachen wie die Infektion mit Viren oder die Benutzung von Mobiltelefonen (Hardell et al., 2012; Lehrer 2012) wird bislang nur spekuliert, weswegen Maßnahmen zur Vorbeugung schlecht anwendbar oder zu empfehlen sind. Lediglich eine hohe Strahlendosis ist als Auslöser belegt, dies würde allerdings nur nach atomaren Unfällen auftreten. Eine

Ausnahme ist hierbei ein Auftreten von GBM als Spätfolge von Radiotherapie anderer Tumore im Kopfbereich, was vor allem bei der Behandlung von Kindern relevant ist (Madden et al., 2010).

Glioblastome zeigen an sich schon eine hohe Heterogenität sowie komplexe Unterschiede in Differenzierungsgraden und Mutationen (Bai et al., 2011). Dies bedeutet auch eine hohe Diversität zwischen den GBMs einzelner Patienten, was es schwierig macht, einzelne spezifische Targets zu identifizieren und daraus einen Therapieansatz zu entwickeln. Lediglich die erhöhte Vaskularisierung und endotheliale Proliferation scheint bei allen GBMs relativ konserviert zu sein (Kaur et al., 2004). Unterschiedliche Mutationen können z.B. bei den Tumorsuppressorgenen wie p14ARF, p16CDKN2A, TP53 und PTEN auftreten (Holland et al., 1998), was zu einer unkontrollierten Proliferation der so entarteten Zellen führt. PTEN (Phosphatase- und Tensin- Homolog) z.B. ist eine Phosphatase, die den PI3K-AKT-mTOR-Signalweg reguliert. Die Aktivierung des Signalwegs führt u.a. dazu, dass über die Bindung von AKT an BAX Apoptose verhindert, über die Aktivierung von mTOR die Translation verstärkt und über eine Ubiquinitierung von FOXO eine unkontrollierte Zellteilung ermöglicht wird (McCubrey et al., 2012). Auch Mutationen von Onkogenen wie EGFR, CDK4 und MDM2 treten häufig auf und können in einer Überaktivierung resultieren (Zundel et al., 2000). Die Mutation von EGFR (*epidermal growth factor receptor*) ist eine der häufigsten Veränderungen von GBM-Zellen und führt zu einer konstitutiven Aktivierung des Rezeptors. EGFR gehört zu den Rezeptor-Tyrosinkinasen (RTK) und aktiviert intrazelluläre Signalwege wie den PI3K-AKT- oder MAP-Kinase-Weg (Taylor et al., 2012). Eine EGFR-Mutation tritt am häufigsten bei primären GBMs auf, während der Verlust der p53-Funktion häufiger bei sekundären GBMs vorkommt. Eine Resistenz gegen auf EGFR abzielende Wirkstoffe kann z.B. durch weitere Punktmutationen, Ko-Aktivierung oder Amplifikation anderer RTKs oder auch Hochregulierung membranständiger Effluxpumpen wie z.B. ABCG2 erfolgen (Taylor et al., 2012). ABCG2 ist ein ATP-bindendes Transportermolekül, das in der Plasmamembran liegt und funktionale Homodimere bildet. Es schleust aktiv verschiedene hydrophobe Stoffe aus der Zelle, zu denen auch zytotoxische Stoffe oder Giftstoffe aus der Nahrung gehören. Effluxpumpen wie ABCG2 tragen nicht nur zur Resistenz gegen zytostatische Wirkstoffe bei, sondern tragen auch zu deren Absorption, Verteilung und Ausscheidung bei (Li et al., 2007). Auch die sogenannten Tumorstammzellen (*cancer stem cells*, CSC) können verschiedene genetische Hintergründe besitzen und sich dementsprechend im Hinblick auf DNA-Reparatur, Zellzyklus-Checkpoints oder Apoptoseinduktion unterschiedlich verhalten. Dies resultiert in einer breiten Diversität nicht nur der differenzierten GBM-Zellen, sondern auch in deren Vorläuferpool und somit zu weiterer genomischer Instabilität (Perez-Garcia et al., 2012) (Abb. 2).

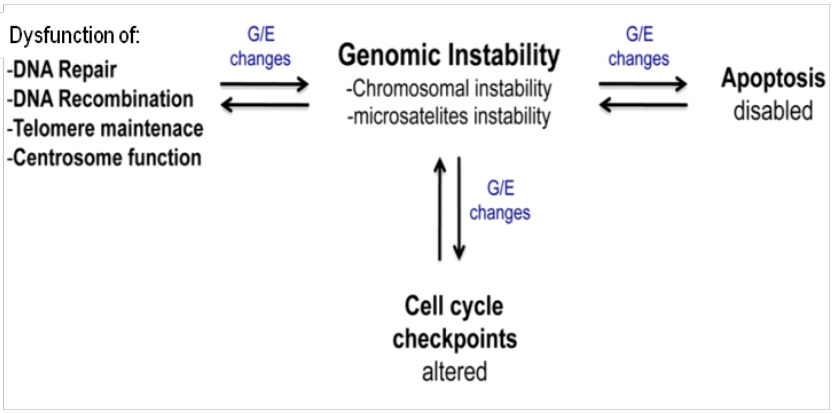

Abb. 2: Darstellung wichtiger genetischer (G) und epigenetischer (E) Mechanismen, die die genomische Instabilität beeinflussen. Durch die genetischen Veränderungen werden auch funktionelle Vorgänge beeinflusst. Aus Perez-Garcia et al., 2012.

1.2 Therapieoptionen

1.2.1 Heutige Standardtherapie

Der heutige Goldstandard in der GBM-Therapie besteht in einer Resektion des größtmöglichen Tumorvolumens und anschließender Röntgenbestrahlung über 6 Wochen mit einer Gesamtdosis von 60 Gy und gleichzeitiger Gabe von Temozolomid (Stupp et al., 2005; Hart et al., 2008). Die synergistische Wirkung von Röntgen-Strahlung und TMZ sowie eine signifikant längere Überlebenszeit von GBM-Patienten wurden durch diese Studie belegt und stellte somit eine Verbesserung zur vorherigen Therapie mit Procarbazin dar (Yung et al., 2000). Durch Bestrahlung wird die DNA proliferierender Zellen so stark geschädigt, dass die Zellen ihre Teilung stoppen und stattdessen in einen programmierten Zelltod gehen. Temozolomid (TMZ) ist ein DNA-alkylierender Stoff, der dadurch Zellen gegenüber ionisierender Strahlung weiter sensibilisiert, da er durch die Methylierung zusätzliche Schäden an der DNA setzt. Es wirkt analog zu Dacarbazin, muss aber im Gegensatz dazu nicht metabolisch in den aktiven Wirkstoff MTIC (Monomethyl-Triazen-Imidazol-Carboxamid) umgewandelt werden (Newlands et al., 1992) (Abb. 3). Dies geschieht bei TMZ automatisch bei physiologischem pH (Bull und Tisdale, 1987). TMZ setzt Methylierungen an unterschiedlichen Stellen der DNA, jedoch wird diejenige an der O^6-Position von Guanin als die kritischste für die Zelle angesehen (Newlands et al., 1997) (Abb. 9). Die Methylierung führt im nächsten Zellzyklus zu DNA-Mismatches mit Thymidin und somit zum Zelltod (Ochs und Kaina, 2000).

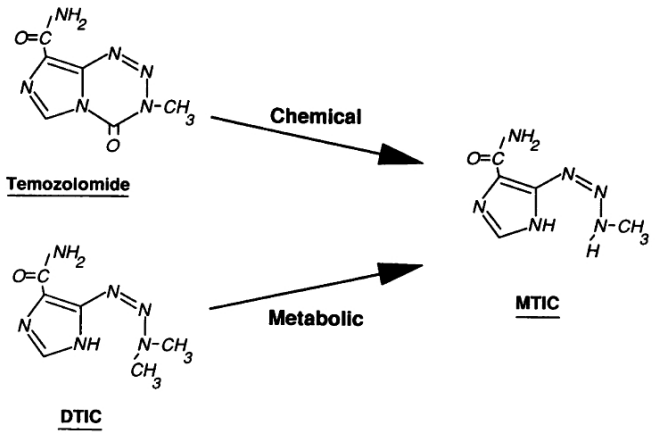

Abb. 3: Struktur von Temozolomid und Dacarbazin (DTIC, Dimethyl-Triazen-Imidazol-Carboxamid) und deren unterschiedliche Umwandlung in den aktiven Wirkstoff MTIC (Monomethyl-Triazen-Imidazol-Carboxamid). Aus Newlands et al., 1992.

1.2.2 Konventionelle Radiotherapie

Röntgen- oder auch Photonenstrahlung ist ungeladene elektromagnetische Strahlung (Jereczek-Fossa et al., 2006), die in der Lage ist, Materie, die sie durchdringt, zu ionisieren. Durch die Interaktion entstehen hochreaktive freie Radikale sowie freie Elektronen, die dann mit Molekülen der Umgebung reagieren und dabei z.B. hydrostatische Bindungen oder auch Strukturen wie die DNA zerstören (Smith und Kao, 2004). Die Schädigung der DNA kann auch auf direktem Wege erfolgen und resultiert in Einzelstrang- oder den schwerwiegenderen Doppelstrangbrüchen (*double strand breaks*, DSBs; Abb. 4). Häufig werden auch Mitochondrien oder Proteine durch die Ionisierung bzw. die resultierenden reaktiven Sauerstoffspezies geschädigt, was zu Fehlfunktionen oder Mutationen führen kann (Zabbarova und Kanai, 2008).
Der therapeutische Effekt von Radiotherapie mit ionisierender Strahlung beruht darauf, dass sich Tumorzellen häufig teilen und dadurch oft ihre DNA replizieren müssen (Helleday et al., 2007; Kastan und Bartek, 2004). Die Zellen versuchen die durch die Bestrahlung gesetzten DNA-DSBs zu reparieren, und bei Erreichen des nächsten Zellzyklus-Checkpoints (zwischen G1- und S-, intra-S- oder G2- und M-Phase, Sancar et al., 2004) entscheidet sich, ob die Zellteilung fortschreitet oder ob der programmierte Zelltod eingeleitet wird.

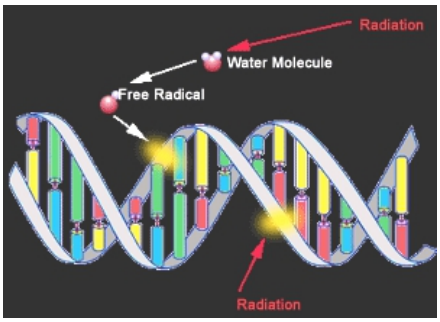

Abb. 4: Wirkung ionisierender Strahlung auf DNA. Die Schädigung kann entweder direkt oder indirekt über reaktive Sauerstoffspezies erfolgen. Photonenstrahlung verursacht Einzelstrangbrüche oder einzelne Doppelstrangbrüche. Quelle: *Space Radiation Analysis Group, Johnson Space Center*

Eine langfristige Nebenwirkung der Strahlentherapie ist jedoch die Schädigung der neuronalen Vorläuferzellen in der subventrikulären Zone oder des Hippocampus. Da Vorläuferzellen in der Lage sind, sich zu teilen und dadurch in begrenztem Umfang neuronale Zellen zu ersetzen, sind sie auch einer Schädigung durch Bestrahlung ausgesetzt (Borges et al., 2008). Dies führt zu kognitiven Ausfällen oder sogar IQ-Verminderung gerade bei Kindern oder auch Embryonen (Marazziti et al., 2012; Andres-Mach et al., 2008). Deshalb versucht man, kritische Bereiche des Hirns bei der Strahlentherapie auszusparen und die größtmögliche Dosis begrenzt im Tumorgewebe zu deponieren. Dies wird durch moderne Methoden wie die intensitätsmodulierte Radio-Therapie (IMRT) realisiert. Hierbei werden zum einen mehrere Einstrahlwinkel gewählt, um nur im Zentrum eine hohe Dosis zu akkumulieren, und zum anderen Abschirmungen verwendet, die speziell auf den Tumor eines Patienten zugeschnitten sind und Strahlung von sensiblen Bereichen abhalten. Photonenstrahlung hat die Eigenschaft, ihre Energie sofort abzugeben, wenn sie mit Materie in Kontakt kommt. Das bedeutet, dass man um in einem tiefer im Gewebe liegenden Tumor genug Energie zu deponieren auch zwangsläufig das weiter außen liegende Gewebe schädigt. Dies wird durch die IMRT zwar vermindert, aber nicht vermieden.
Bei gesunden Zellen, die durch ionisierende Strahlung geschädigt werden, ist bekannt, dass sie eine genomische Instabilität erwerben können, die nach einigen weiteren Zellzyklen zu einer malignen Transformation führen kann (Wright und Coates, 2006; Shah et al., 2012). Es besteht der Verdacht, dass dies auch schon nach therapeutischen Dosen vorkommen kann. Gesunde Zellen des ZNS wie z.B. Neurone, Gliazellen oder neuronale Vorläuferzellen sind ebenso betroffen von der ionisierenden Wirkung der Bestrahlung, jedoch befinden sie sich je nach Differenzierungsstatus seltener oder gar nicht mehr im Zellzyklus. Adulte Neuronen sind normalerweise nicht mehr teilungsfähig und bleiben in der G0-Phase. Sie reparieren entweder ihre DSBs, was zur normalen Proteinbiosynthese beiträgt, oder aber sie akkumulieren die Schäden und erzeugen somit mutierte Proteine (Abb. 5) (Fishel et al., 2007).

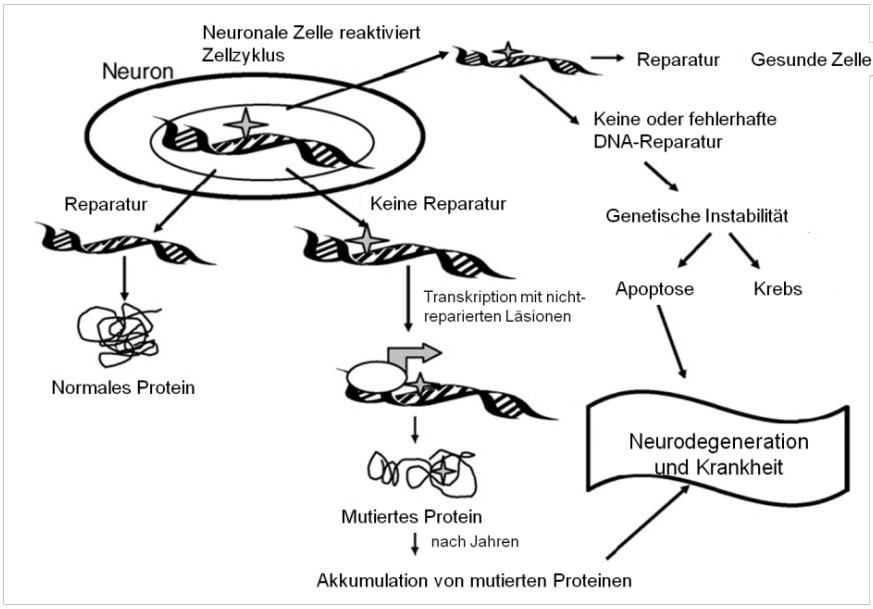

Abb. 5: DNA Doppelstrangbrüche in adulten Neuronen werden entweder repariert oder bleiben bestehen, was zu Neurodegeneration oder Zelltod führen kann. Modifiziert nach Fishel et al., 2007.

Ein zusätzlicher Schutz für Neurone besteht in der engen Nachbarschaft zu Gliazellen wie den Astrozyten, die die Neurone z.B. durch Aufnahme von überschüssigem Glutamat oder Wasserstoffperoxid (H_2O_2) schützen (Tofilon und Fike, 2000).

Als Alternative zur Photonentherapie wird seit Kurzem eine Partikelbestrahlung angewandt (Durante und Löffler, 2010). Ideal eignen sich dafür Kohlenstoff-(C-) Ionen wegen ihrer physikalischen Eigenschaften (Schulz-Ertner und Tsuji, 2007; Ando und Kase, 2009; Blakeley und Chang, 2009; siehe Abb. 6 sowie Kap. 1.2.3). Auch die Protonenbestrahlung wird in letzter Zeit vermehrt als Therapieoption in Betracht gezogen, da Wasserstoffionen technisch gesehen auch zur Partikeltherapie gehören. Allerdings sind Protonen auf Grund ihrer kleinen Masse sehr leicht und haben eher die Eigenschaften von Photonen im Vergleich zu den schwereren Ionen wie Kohlenstoff, Neon oder Sauerstoff (Jäkel, 2009). Protonenbeschleuniger sind jedoch einfacher zu bauen und die Behandlung weniger kostenintensiv.

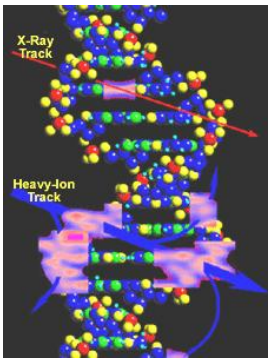

Abb. 6: Schwerionen-Strahlung verursacht an DNA-Molekülen oft mehrere DSBs dicht nebeneinander (Cluster-Schäden), die für Zellen sehr schwer zu reparieren sind. Quelle: *Space Radiation Analysis Group, Johnson Space Center*

1.2.3 Schwerionentherapie mit Kohlenstoffionen

Schwerionen wie auch Protonen und Neutronen gehören zur Gruppe der Hadronen und sind Partikel, deren Elektronen durch physikalische Methoden entfernt wurden. Als Schwerionen (SI) werden Partikel bezeichnet, die eine größere Masse als Helium besitzen. Charakteristisch für SI ist die Energieabgabe in einem scharf begrenzten Maximum in der Tiefe von Materie, dem sogenannten Bragg Peak (Tsuji und Kamada, 2012; Hamada et al., 2010). Dieses inverse Tiefendosisprofil entsteht durch die Interaktion der Ionen mit dem Gewebe, durch das sie sich bewegen. Zunächst ist die Energie der Ionen hoch und die Interaktion gering. Mit der Strecke, die sie im Gewebe zurücklegen, verlieren sie jedoch an Geschwindigkeit, und somit erhöhen sich die Interaktionen und die Energie wird geringer (Abb. 7). Dies führt zu einem maximalen Energietransfer und damit einer peakförmigen Dosisdeposition am Ende der zurückgelegten Strecke (Kraft 1998; Schulz-Ertner et al., 2006).

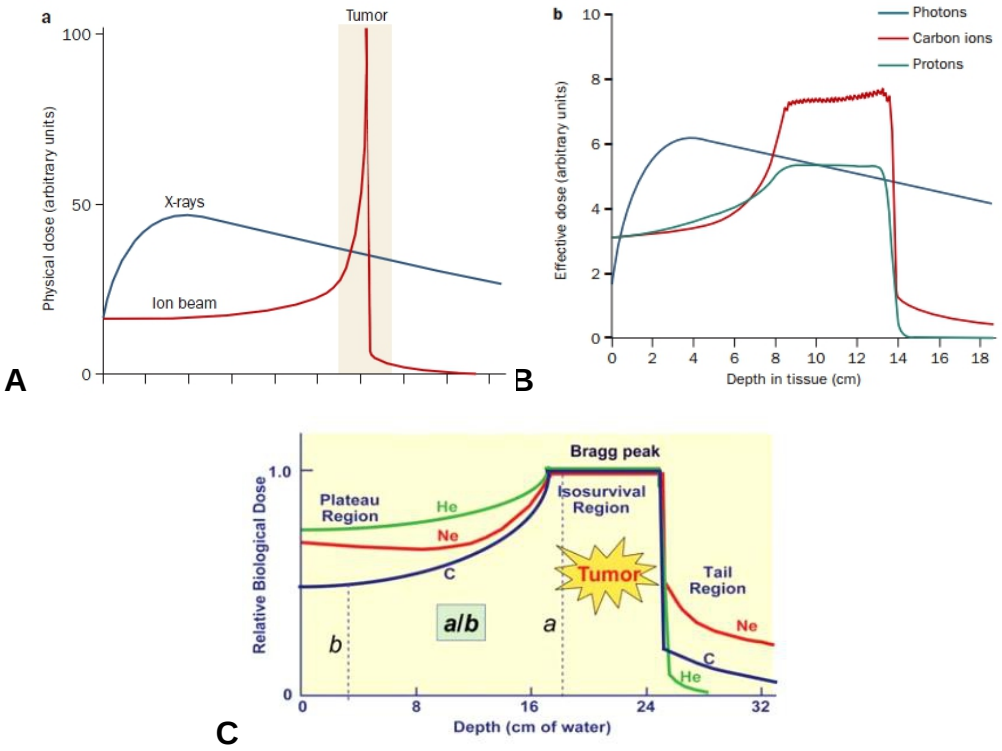

Abb. 7: Wechselwirkung von Photonen- und Ionenstrahlung mit Materie. A: Physikalische Dosisabgabe von Photonen und Kohlenstoff-(C-) Ionen in Gewebe. C-Ionen geben im Eingangskanal nur wenig Energie ab, während Photonen schon in geringer Tiefe viel Energie deponieren und dadurch das Gewebe schädigen. Die maximale Energiedeposition von C-Ionen erfolgt im Bragg Peak. B: Unterschiedliche Energiedeposition von Photonen-, Protonen- und Kohlenstoffionenstrahlung in der Tiefe von Gewebe. Die Bragg Peaks von Partikelstrahlen können durch verschiedene Eingangsenergien ausgedehnt werden (*spread out Bragg Peak*, SOBP). A und B aus: Durante und Löffler, 2010. C: Energiedeposition im ausgedehnten Bragg Peak (SOBP) verschiedener Ionen im Tumorgewebe. Kohlenstoffionen geben dabei am wenigsten Energie an umliegendes Gewebe ab. Aus Tsuji und Kamada, 2012.

Im Gegensatz zu Photonenstrahlung besitzt ein SI-Strahl einen hohen linearen Energietransfer (LET), der dazu führt, dass die Schäden an der DNA dichter beieinander liegen und damit schwerer zu reparieren sind (Cluster-Schäden, Asaithamby et al., 2011; Durante und Löffler, 2010). Außerdem erfolgt die Schädigung durch SI unabhängig von Sauerstoffmolekülen und kann somit auch z.B. Tumorzellen in hypoxischen Bereichen abtöten. Diese Effekte werden als erhöhte relative biologische Wirksamkeit (RBE) zusammengefasst, was bedeutet, dass SI bei gleicher applizierter Dosis gegenüber Photonen eine stärkere Wirkung auf das Zielgewebe haben. Der Faktor variiert je nach Ionentyp und Eingangsenergie (Hamada et al., 2010; Ando und Kase, 2009). Ein weiterer Vorteil der Ionenbestrahlung ist eine Inaktivierung der radioresistenteren Tumorstammzellen sowie der Zellen, die sich gerade nicht im Zellzyklus befinden (Jereczek-Fossa et al., 2006). Für eine SI-Therapie sind verschiedene Eigenschaften der Ionen wichtig. Ideal ist ein scharf abgrenzbarer Bragg Peak, eine niedrige Energiedeposition im Eingangskanal und eine hohe RBE bei gleichzeitiger maximaler Schonung des gesunden Gewebes (Schulz-Ertner und Tsuji, 2007). Dies alles ist bei Kohlenstoffionen kombiniert, die als einziger Ionentyp noch die Eigenschaft haben, bei ihrer Energieabgabe im Gewebe Positronen auszusenden, die man mit Hilfe einer PET-Kamera detektieren kann. Somit ist während einer Therapiesitzung eine direkte Überwachung der Bestrahlung im Gewebe möglich (Pönisch et al., 2004).
Für die Behandlung eines Tumors als dreidimensionales Volumen ist ein einzelner Bragg Peak allerdings nicht von therapeutischem Nutzen. Deshalb wurde eine Methode entwickelt, den Peak durch verschiedene Eingangsenergien des Ionenstrahls räumlich so auszudehnen, dass ein Energie-Plateau im Zielgewebe entsteht. In der Therapieplanung wird das 3D Tumorvolumen in einzelne „Scheiben“ unterteilt, von denen jede unterschiedlich geformt sein kann. Diese werden dann nach und nach durch ein Rasterscanverfahren Punkt für Punkt angesteuert und bestrahlt (Haberer et al., 1993) (Abb. 8). Dadurch wird eine hohe Energieabgabe im Tumorgewebe erreicht, während umliegendes, sensitives Gewebe maximal geschont wird. Dieses Prinzip wird Dosis- Konformation genannt (*dose conformation*, Jäkel, 2009).

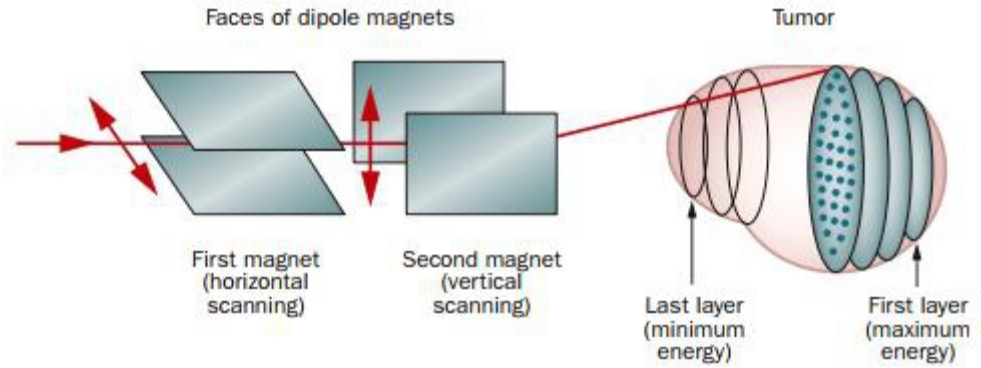

Abb. 8: Das Prinzip des intensitätsgesteuerten Raster-Scanning-Verfahrens. Das Tumorvolumen wird in unterschiedliche „Scheiben“ eingeteilt und jede davon mit einer anderen Eingangsenergie des Kohlenstoffstrahls angesteuert. Aus Durante und Löffler, 2010.

Dieses Verfahren wird sehr erfolgreich bei abgegrenzten Tumoren im Kopf- und Halsbereich angewendet (Schulz-Ertner et al., 2007). In Deutschland wurde hierfür das neue Heidelberger Ionenstrahl-Therapiezentrum (HIT) gebaut. Die Forschung und Entwicklung wurde bei der GSI Helmholtzzentrum für Schwerionenforschung in Darmstadt durchgeführt. Zurzeit wird erforscht, ob diese Art der Bestrahlung auch für andere Tumorarten bzw. für bewegliche Organe wie Lunge oder Herz anwendbar werden kann. Zur GBM-Therapie laufen bereits klinische Studien (Combs et al., 2010a, b).

1.3 Neue Therapieansätze

Da die Heterogenität von GBM und auch das unterschiedliche Ansprechen der Patienten auf die Standardtherapie bekannt sind, wird aktuell intensiv nach neuen Methoden gesucht, um die Therapie effektiver zu gestalten. Viele Mutationen in GBM lassen sich derzeit aus Biopsien oder Primärkulturen nachweisen und werden in präklinischen Experimenten untersucht (Szerlip et al., 2012; Liu et al., 2013; Johnson et al., 2012). Allerdings ist bislang noch wenig über ihren Einfluss auf den Verlauf einer GBM-Erkrankung und das Überleben der Patienten bekannt. Somit ergibt sich derzeit keine Konsequenz aus der Bestimmung von Mutationen für eine Therapie, da entsprechende Wirkstoffe noch nicht verfügbar sind. Der zurzeit einzige klinisch angewandte prädiktive Parameter ist die Bestimmung der Methylierung des Promotors der O^6-Methylguanin-DNA- Methyltransferase (MGMT). Ist der Promotor nicht methyliert, wird das Enzym kontinuierlich exprimiert und kann somit die Methylierungen, die durch TMZ an der DNA entstehen, wieder rückgängig machen (Skiriute et al., 2012; Nakada et al., 2012) (Abb. 9).

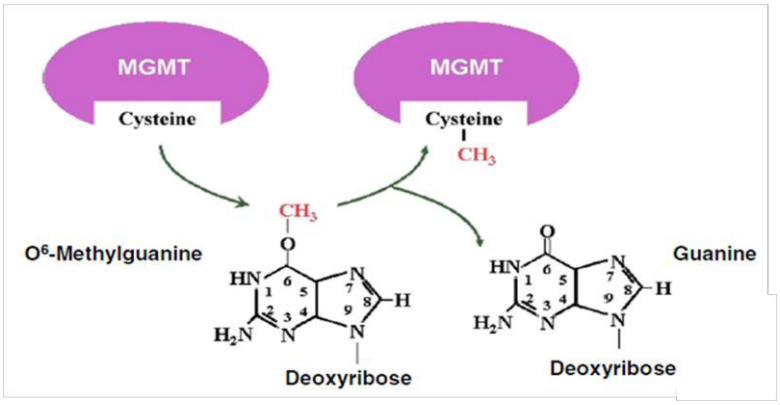

Abb. 9: Wirkweise der O^6-Methylguanin-DNA-Methyltransferase (MGMT). MGMT entfernt Methylierungen der DNA an der O^6-Position von Guanin, die z.B. durch TMZ entstehen. Das Enzym selbst degradiert nach der Reaktion, weshalb die Rate, mit der O^6-Methylierungen entfernt werden, auch als Nachweis der Neubildung des Enzyms gewertet wird. Aus Silber et al., 2012.

Humane Zellen können nur mit Hilfe des Enzyms MGMT zytotoxische O^6-Alkylguaninreste der DNA entfernen. In normalen Zellen agiert es als Tumorsuppressor, da es DNA-Schäden und somit Mutationen verhindert. In Tumorzellen jedoch, bei denen man mit der Behandlung genau diese DNA-Schäden setzen möchte, vermittelt es die Resistenz gegenüber TMZ oder anderen alkylierenden Wirkstoffen (Silber et al., 2012). Es besteht die Vermutung, dass Patienten mit demethylierter Promotorsequenz eine schlechtere Prognose für die Therapie mit TMZ haben (Hegi et al., 2005), allerdings ist diese Vermutung nicht abschließend bestätigt, und die meisten Patienten erhalten standardmäßig trotzdem die Radio-Chemotherapie nach dem Stupp-Protokoll (siehe Kap. 1.2.1) (Stupp et al., 2005; Hart et al., 2008).

Neben der Entwicklung neuer Bestrahlungsmethoden und dem Finden wirksamer Chemotherapeutika gibt es Hinweise darauf, dass die effektive Abtötung der heterogenen Zellpopulation in GBM nur durch eine Kombination von Wirkstoffen erfolgreich sein wird. Bei Behandlung mit nur einem Wirkstoff ist bekannt, dass Tumorzellen alternative Signalwege aktivieren, um einen zytotoxischen Effekt zu vermeiden und somit ihre Vermehrung zu sichern (Taylor et al., 2012; Bai et al., 2011) (Abb. 10). Aktuelle Studien befassen sich mit der Aufklärung der vielfältigen Tumorsignalwege, um einen Weg zu finden, die unkontrollierte Proliferation der Zellen zu unterbinden. Mögliche Zielmoleküle sind dabei EGF (*epidermal growth factor*), VEGF (*vascular Endothelial Growth Factor*), SHH (*sonic hedgehog*) oder auch PDGF (*platelet-derived growth factor*) (Bai et al., 2011; Lima et al., 2012). Ziel der Experimente ist es, z.B. über siRNA oder spezifische Antikörper Moleküle zu blockieren und somit in das Tumorsignalling einzugreifen. VEGF zum Beispiel kann über Bevacizumab alleine oder in Kombination mit Irinotecan inhibiert werden. Eine klinische Studie darüber zeigte jedoch, dass nur eine Untergruppe der Patienten wirklich von dieser Art der Behandlung profitierte (Rahman et al., 2010), weshalb diese Therapie nicht weiter angewendet wurde.

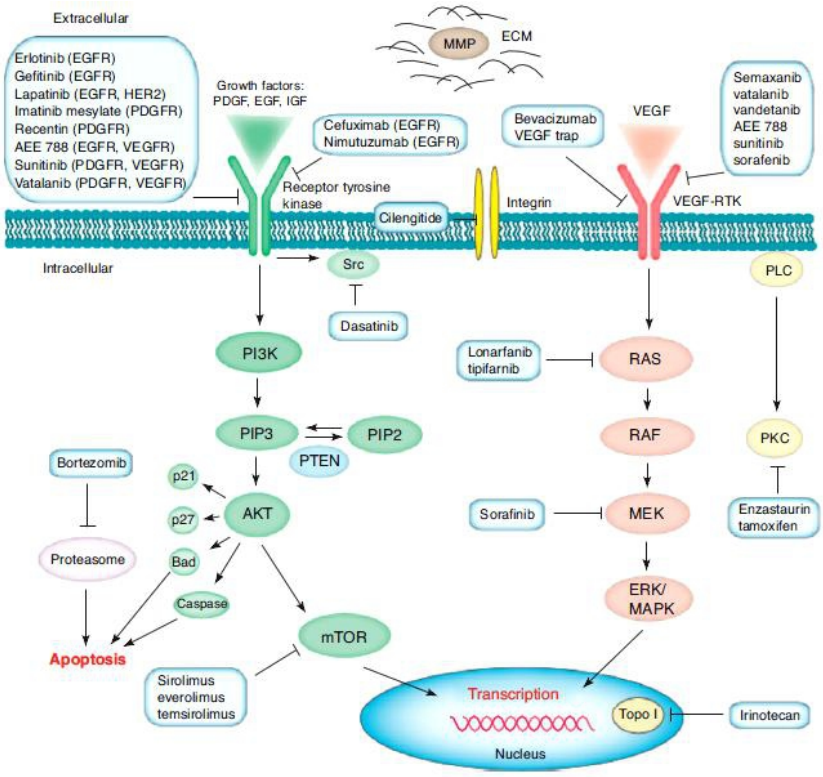

Abb. 10: Übersicht über molekulare Targets in GBM, die derzeit untersucht werden. Oft sind dies onkogene RTK-Signalwege wie PI3K-AKT (grün) oder RAS (pink), die über intrazelluläre Inhibitoren beeinflusst werden. EGF, VEGF oder auch PDGF werden durch Moleküle oder monoklonale Antikörper geblockt. In blauen Boxen sind Wirkstoffe angegeben, die die jeweiligen Signalwege unterbinden. ECM- extrazelluläre Matrix, MMP- Matrixmetalloproteinasen. Aus Bai et al., 2011.

Auch die Tumorstammzellen (CSC) stellen eine eigene Population dar und reagieren anders auf Bestrahlung und Zytostatika als die ausdifferenzierten Tumorzellen (Beier et al., 2011). Sie werden darüber definiert, dass sie CD133 exprimieren, und nur diese Subpopulation ist in der Lage, einen Tumor zu propagieren, wenn sie in Nacktmäuse transplantiert werden (Singh et al., 2004; Galli et al., 2004). Allerdings wird kontrovers diskutiert, ob die Expression von CD133 als alleiniges Kriterium zur Identifikation von CSC ausreicht, da es Hinweise auf weitere Subpopulationen innerhalb der CSC gibt, die nicht unbedingt CD133+ sind (Wan et al., 2010; Chen et al., 2010; Günther et al., 2008). Sie liegen oft in sogenannten „Nischen“, die durch extrinsische Faktoren der lokalen Mikroumgebung wie umliegende Vorläufer- und differenzierte Zellen, Zell-Zell-Interaktionen, Zilien, lösliche Faktoren oder Neurotransmitter definiert sind (Faigle und Song, 2013). Zudem proliferieren CSC weniger als die differenzierten Tumorzellen und sind deshalb durch die gängigen Therapien schwer angreifbar (Jamal et al., 2012).

Es wurde auch gezeigt, dass Tumorzellen und CSC in Xenografen, also innerhalb einer intakten Mikroumgebung (*Microenvironment*), wesentlich radioresistenter sind als *in vitro*

bzw. als Monolayer oder Neurosphärenkultur (Ropolo et al., 2009; Jamal et al., 2012). Nach einer Radiotherapie haben die CSC also eine höhere Überlebenswahrscheinlichkeit und sind somit vermutlich für eine spätere Rekursion des Tumors verantwortlich sind (Tamura et al., 2010).

Die Chemoresistenz von CSC gegenüber TMZ ist noch nicht geklärt. Verschiedene Studien, die in den letzten Jahren erschienen sind, kommen zu unterschiedlichen Ergebnissen (Beier et al., 2011). Die Sensibilität der CSC scheint vom Ursprung der Zellen (Zelllinie, tierischen oder humanen Ursprungs, oder aus Primärtumoren), den Wachstumsbedingungen (in serumfreiem Medium oder als Neurosphären), aber auch von einer Selektion der Zellen nach CD133-Expression oder MGMT-Methylierung abzuhängen. Auch wenn in einzelnen *in vitro* Studien eine vollständige Inaktivierung der CSC erreicht werden konnte (Beier et al., 2008), steht dem gegenüber die Beobachtung, dass Patienten nach TMZ-Therapie fast immer ein Rezidiv erleiden.

Die Resistenz der CSC könnte intrinsisch oder extrinsisch vermittelt sein (Abb. 11), was in beiden Fällen zu einem erneuten Tumorwachstum führt, aber auf unterschiedliche Weise behandelt werden müsste.

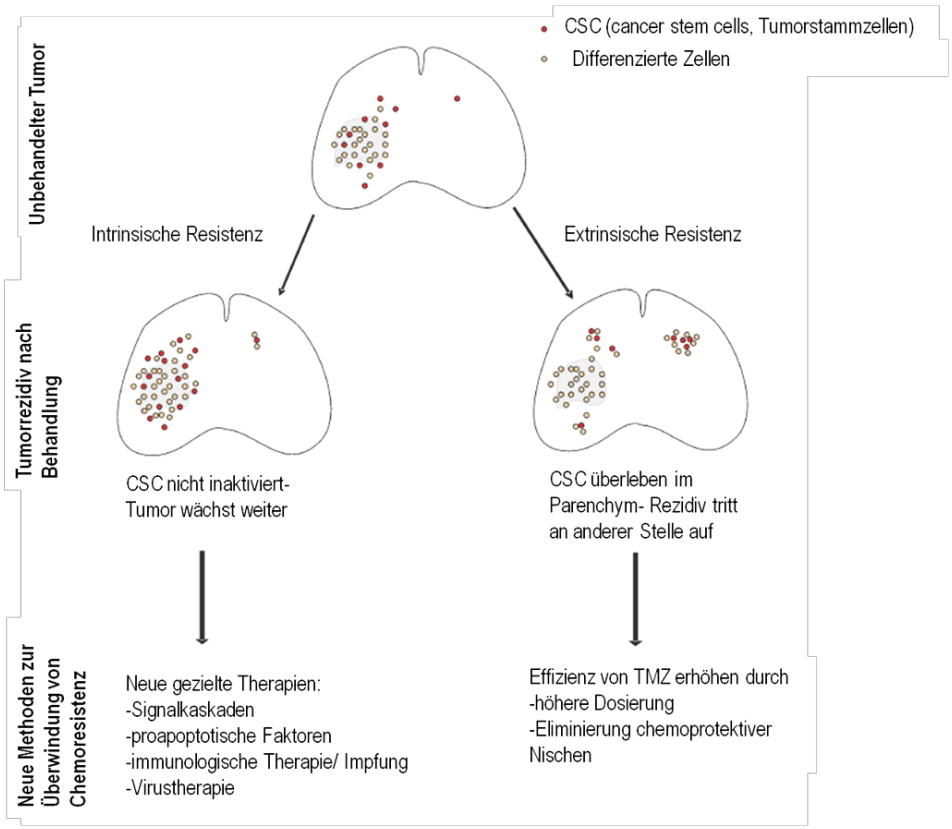

Abb. 11: Durch intrinsische Resistenz von CSC in GBM kann es zu einem Rezidiv des Tumors an der Stelle des Primärtumors oder auch an einer anderen Stelle durch Invasion kommen. Extrinsische Resistenz sorgt für den Schutz invasiver Zellen ins Parenchym, während alle Zellen in der ursprünglichen Tumormasse abgetötet werden. Modifiziert nach Beier et al., 2011.

Intrinsische Resistenz könnte durch MGMT- Expression vermittelt werden, aber es ließe sich dann nur schwer erklären, warum es auch Rückfälle von Patienten ohne diese Mutation gibt (Hegi et al., 2005). Die Rolle von Multiresistenz-vermittelnden Proteinen wird ebenfalls kontrovers diskutiert, da nicht klar ist, ob TMZ wirklich durch diese Proteine aus der Zelle transportiert wird (Schaich et al., 2009). Ein weiterer Mechanismus der Resistenzbildung könnte eine verbesserte DNA-Reparatur sein. Die Wirkung von TMZ über die Methylierung der DNA führt am ehesten zu toxischen Doppelstrangbrüchen, wenn die Zellen eine Mismatch-Reparatur durchführen. Gibt es in diesem System Mutationen (z.B. an der wichtigen Mismatch-Komponente mut6 homolog6, MSH6; Yip et al., 2009), könnten diese durchaus zu einer TMZ-Resistenz führen. Auch die Aufhebung der Zelltodinduktion durch DNA-DSBs durch Mutationen in Proteinen wie p53 oder PARP (Poly-ADP-Ribose-Polymerase) oder Mutationen direkt im Apoptose-Signalweg könnten zu einer Chemoresistenz beitragen (Roos et al., 2007), sind aber bislang wenig charakterisiert.
Die extrinsische Resistenz wird hauptsächlich von Bestandteilen der Blut-Hirn-Schranke (*blood- brain- barrier*, BBB) gebildet, die im gesunden Parenchym dafür sorgen, dass die Konzentration an Wirkstoff geringer ist als im restlichen Körper. Im Tumorgewebe selber kann die BBB gestört sein und somit wirksame Konzentrationen des Zytostatikums erreicht werden, allerdings werden die invasiven Zellen im umliegenden gesunden Gewebe nicht genug geschädigt, da hier die intakte BBB die Konzentration des Wirkstoffs zurückhält (Rosso et al., 2009; Beier et al., 2011). Außer den BBB-Komponenten spielen auch direkte Zell-Zell-Interaktionen, lokale Sekretion von Zytokinen wie IL-6 oder SDF-1, oder auch Faktoren wie Hypoxie eine Rolle in der Resistenzbildung (Meads et al., 2008). Eine Hochregulation von STAT-3 kann ebenfalls zur TMZ-Resistenz der CSC in GBM beitragen (Villalva et al., 2011).
All diese Faktoren machen deutlich, dass es schwierig ist, die eine wirksame Therapie gegen GBM zu entwickeln. Es wird immer klarer, dass es wichtig ist, jedes GBM vor einer Therapie so detailliert wie möglich zu charakterisieren und somit eine personalisierte Therapie zu entwerfen. Im Folgenden sollen Stand der Forschung und neue Modelle dargestellt werden, die eine GBM Therapie effektiver machen könnten.

1.4 Aktueller Stand der Forschung

Präklinische GBM-Forschung wird zumeist an Zelllinien oder Tiermodellen durchgeführt. Zellkulturen haben den Vorteil, dass sie relativ leicht zu halten und kostengünstig sind. Man verwendet entweder meist über Jahre etablierte und subkultivierte Zelllinien oder initiiert primäre Linien aus Tumorbiopsien, die von Patienten oder in Tieren gewachsenen Tumoren stammen können. Vorteile der Zellkulturen sind das schnelle Wachstum der Zellen, die

Vermehrung durch Subkultivierung und dadurch verminderte Schwankungen der Ergebnisse bei Wiederholung der Experimente. Gerade durch die Subkultivierung ergeben sich aber auch Nachteile. Mit steigender Zeit in Kultur findet zwangsläufig eine Selektion der Zellen statt, die sich besser an die Kulturbedingungen anpassen. Häufiger proliferierende Zelltypen werden sich ebenfalls stärker in einer Kultur durchsetzen, und die Zellen zeigen eine erhöhte genomische Instabilität, was die Ähnlichkeit zum Originaltumor verringert (Perez-Garcia et al., 2012). Bei Zelllinien, die schon jahrelang in Verwendung sind, wie z.B. U-87 MG, SF-767-, oder TG-98G-GBM-Zellen, ist auch ein klonales Verhalten anzunehmen und macht einen Vergleich mit einem primären, heterogenen GBM fast unmöglich. Zellkulturen vernachlässigen zudem völlig den Aspekt, dass der Tumor im Organismus in Wechselwirkung mit der extrazellulären Matrix bzw. dem *Microenvironment* sowie dem Immunsystem und der Blutversorgung steht.

Bei Tiermodellen soll eine natürlichere Umgebung für das Tumorwachstum hergestellt werden. Man unterscheidet hierbei spontane Onkogenese oder Xenografte, die auf verschiedene Arten implantiert werden können (Sonabend et al., 2007; Jacobs et al., 2011). Spontane Onkogenese wird z.B. durch die Gabe von N-Methyl-N-nitrosoharnstoff (MNU) über das Trinkwasser erreicht. In einem Modell mit Sprague-Dawley-Ratten entstehen nach bis zu 20-wöchiger Gabe von MNU ca. 40% anaplastische Gliome (Rushing et al., 1998), die sowohl eine astrozytäre als auch oligodendrogliale Komponente zeigen. Da die Forschung für humane GBM-Patienten optimiert werden soll, werden häufig Xenografte eingesetzt, also die Transplantation humaner GBM-Zellen oder -Zelllinien in Mäuse oder Ratten. Um eine Abstoßung aufgrund der Speziesunterschiede zu vermeiden, werden Xenografte oft in immundefizienten, athymischen Nacktmäusen erzeugt. Das Mausmodell „U251“ wurde z.B. durch die Kultivierung von humanen GBM-Zellen eines Patienten und anschließender subkutaner oder intrakranialer Transplantation etabliert (Houchens et al., 1983; Radaelli et al., 2009). Die U251-Zellen haben die wesentlichen Charakteristika eines GBM und zeigen im Xenograft unter anderem Merkmale wie Infiltration, Pseudopalisadenbildung oder Mitosefiguren. Außerdem beinhalten U251-Xenografte eine CD133-positive „Stammzell“population. Ein großer Nachteil dieses Modells ist allerdings das Fehlen der immunologischen Komponente.

Ein Beispiel für ein syngenes Modell unter Berücksichtigung des Immunsystems ist das GL261-Modell in C57BL/6- Mäusen (Seligman und Shear, 1939). Hierfür wurden zunächst Tumore durch Implantation von Methylcholantren in das Hirn von Mäusen erzeugt. Dieses Gewebe wurde entnommen und zu Zellkulturen verarbeitet. Die so erhaltenen Zelllinien erzeugen bei erneuter Transplantation in Hirngewebe von Mäusen GBM-ähnliche Tumore (Zagzag et al., 2000). Diese Tumore zeigen wichtige Eigenschaften von GBM wie Invasion, Pseudopalisadenbildung, Nekrose und Anfärbung mit GFAP.

Bei der Verwendung von Tiermodellen muss man sich also entscheiden, ob man den Schwerpunkt auf die Verwendung humaner Zellen legt und damit näher am menschlichen Tumor bleibt, dafür aber die immunologische Komponente vernachlässigt, oder ob man ein immunkompetentes Modell verwendet, aber dafür größere Speziesunterschiede in Kauf nimmt. Ein zusätzlicher Nachteil von Tierversuchen generell, aber gerade bei Tumormodellen, ist die große Belastung für die Tiere. Je nach Endpunkt des Experiments kann es notwendig sein, das Fortschreiten der Krankheit bis zum Tod des Versuchstiers zu beobachten. Im Zuge der Initiative einer Reduktion von Tierversuchen und der 3R-Regel („*Replace, Refine, Reduce*“ -Ersetzen, Verfeinern und Reduzieren von Tierversuchen; Russell und Burch, 1959) wird versucht, Tierversuche zu ersetzen, die nicht unbedingt nötig sind. Ein Modell, das diese Anforderungen sehr gut erfüllt, ist das sogenannte organotypische hippocampale Slicekultur-Modell nach Stoppini (Stoppini et al., 1991), was seither von vielen Arbeitsgruppen für neurobiologische Experimente verwendet wird. Hierbei wird Hirngewebe neonataler Ratten oder Mäuse mit Hilfe eines Vibratoms in ca. 350 µm dicke Scheiben („Slices“) geschnitten und auf Membranen an einer Grenzfläche zwischen Kulturmedium und Luft kultiviert. Dieses OHSC-Modell erhält die im Hippocampus parallel verlaufenden Fasertrakte zwischen entorhinalem Cortex (EC), Cornu- Ammonis- Region (CA), Gyrus Dentatus (DG) und Subiculum (Sub, Abb. 12) und ermöglicht so eine Langzeitkultivierung der Schnittkulturen, an denen weiterhin Fragestellungen zu Erregungsleitung, neuronaler Schädigung oder Immunreaktion durch Mikroglia untersucht werden können (Kluge et al., 1998; Hailer et al., 2001; Eyupoglu et al., 2003).

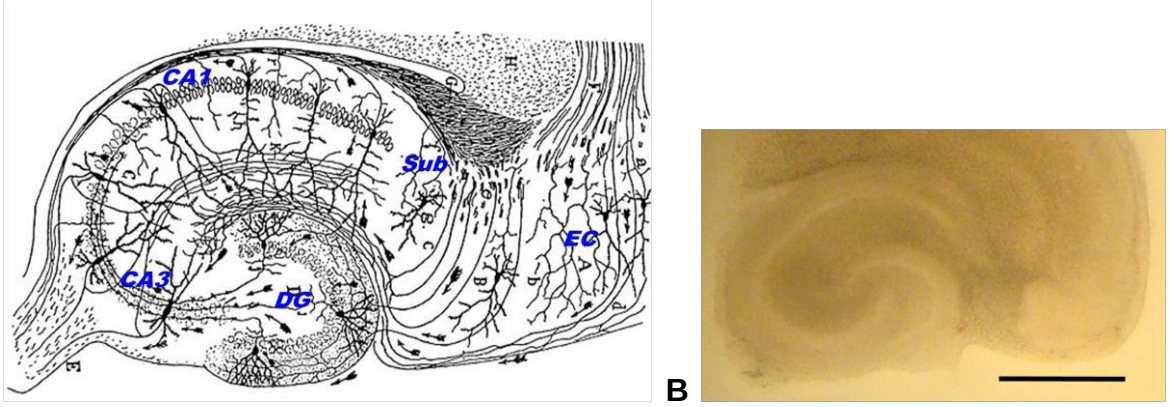

Abb. 12: Hippocampusformation bei Nagetieren. A: Die Reizweiterleitung verläuft entlang der Fasertrakte aus den verschiedenen Schichten des EC in den DG und CA3 und 1, dann aus dem DG nach CA3, von CA3 nach CA1 und von dort aus über das Subiculum wieder in den EC. CA- Cornu Ammonis, DG- Gyrus dentatus, Sub- Subiculum, EC- Entorhinaler Cortex. Modifiziert nach Santiago Ramón y Cajal, 1911. B: Organotypische hippocampale Slicekultur (Foto: S. Kallendrusch; Balken= 1mm).

Dieses System bietet die Möglichkeit, Gewebe unter Erhalt aller beteiligten Zelltypen und der extrazellulären Matrix über längere Zeit zu kultivieren. Es wurde zunächst mit Gewebe von Nagetieren angewendet, jedoch besteht in der präklinischen Forschung die Notwendigkeit, den Speziesunterschied zu überwinden und humanes Gewebe zu verwenden. Bei gesundem Gewebe ist die Gewinnung aus ethischen Gründen limitiert, jedoch wird durch chirurgische Eingriffe bei malignen Erkrankungen oft Tumorgewebe entnommen, das für die Forschung wertvoll ist. Wird aus diesem Gewebe eine Slice-Kultur angelegt, kann man Untersuchungen zur Reaktion auf verschiedene Stimulationen oder Behandlungen anstellen und minimiert inter-individuelle Unterschiede.

Speziesunterschiede stellen ein großes Problem in der präklinischen Forschung dar. Genom und Stoffwechsel von Mäusen und auch Primaten unterscheidet sich substantiell von dem des Menschen. Deshalb kann man erwünschte Wirkungen, aber auch Nebenwirkungen, nicht verlässlich anhand von Tierversuchen voraussagen. Dass Nebenwirkungen auch nach gründlichen Vorversuchen auftreten können, wurde 2006 offensichtlich, als in London der monoklonale CD28 Antikörper (TGN1412) zur Anwendung bei rheumatoider Arthritis oder Leukämie an freiwilligen Versuchspersonen in einer Phase-I-Studie getestet wurde. Diese entwickelten extrem schnell schwere toxische Schockzustände und überlebten nur mit gravierenden Schäden. Es stellte sich heraus, dass es eine geringfügige Veränderung der Aminosäure-Abfolge im Protein der Primaten gab, die dazu führte, dass der Antikörper beim Menschen eine enorme Immunreaktion und einen sogenannten Zytokinsturm auslöste, der zum Multiorganversagen führte. Die klinische Studie wurde daraufhin eingestellt (Kenter und Cohen, 2006; Dowsing et al, 2007). Dies machte umso deutlicher, dass die Notwendigkeit besteht, humane Testmodelle zu etablieren, die die Reaktion im Patienten wiederspiegeln und eine Möglichkeit bieten, diese Vorgänge auch auf molekularer Ebene zu untersuchen. Ansätze dazu gibt es unter anderem von unserer Arbeitsgruppe mit humanem Gewebe aus Tonsillektomien oder GBM- sowie otorhinolaryngologischen Operationen (Merz und Bechmann, 2011). In der Literatur sind bislang nur wenige ähnliche Ansätze zu finden (Vaira et al., 2010; Liu et al., 2011), jedoch scheint sich der Gedanke, Forschung für humane Fragestellungen auch an humanen Systemen zu untersuchen, langsam zu verbreiten (Seok et al., 2013). Im Rahmen dieser Promotionsarbeit wurde in den vergangenen fünf Jahren die Etablierung eines humanen GBM-Slice-Kultur-Modells zur Testung bekannter und neuer Zytostatika in Kombination mit etablierten und neuen Bestrahlungstherapieoptionen erreicht (Müller et al., 2010; Merz et al., 2010; Merz und Bechmann 2011; Merz et al., 2013). Hierbei wird aus Resektionen erhaltenes GBM-Gewebe in Schnitt-Kulturen überführt, die 350 µm dick sind. Somit bleiben in diesen Gewebeschnitten alle Zelltypen sowie auch Endothel und extrazelluläre Matrix erhalten, und in Experimenten an diesen Kulturen sind alle Komponenten des Gewebes bei der Reaktion beteiligt.

Wir konnten zeigen, dass GBM-Slices bis zu zwei Wochen in Kultur ohne signifikanten Zelltod oder morphologische Veränderungen überleben und weiter proliferieren. Behandlung der Schnittkulturen mit Röntgenbestrahlung oder TMZ zeigt ähnliche Effekte wie in Patienten, die nach dem gängigen Standardprotokoll behandelt werden, wie DNA-Schädigung, Verminderung der Proliferation oder Induktion von Zelltod. Dieses Modell könnte es in Zukunft ermöglichen, eine personalisierte Therapie für einen Patienten zu ermitteln, da es aufgrund der vielfältigen Ausprägungen und Mutationen bei GBM nicht unbedingt die eine richtige Therapie für alle Patienten geben muss. Außerdem bietet es eine Plattform für detaillierte Untersuchungen zu molekularen Mechanismen der Tumorresistenz, da man nach Ablauf eines Experiments die überlebenden Zellen charakterisieren kann. Zusätzlich sind Live-Imaging-Setups zur Langzeitbeobachtung einzelner Schnittkulturen machbar.

2. Fragestellung

Während der letzten Jahrzehnte wurde in der Therapie von GBM trotz intensiver Forschung weder im Hinblick auf Pathogenese noch auf die Therapie ein wirklicher Durchbruch erzielt. Die bislang verwendeten Modelle beruhen meist auf Tierexperimenten oder häufig subkultivierten Zellkulturen. Um Speziesunterschiede sowie Artefakte der Zellkultur zu vermeiden, wurden in dieser Arbeit folgende Fragen gestellt:

-Lassen sich Slice-Kulturen aus humanen GBM-Proben gewinnen und über einen längeren Zeitraum kultivieren?

-Kann ein Setup zur Bestrahlung der Slice-Kulturen mit Photonenstrahlung aufgebaut werden, so dass Vitalität und Sterilität der Kulturen gewährleistet bleiben?

-Können die Slice-Kulturen später auch mit Kohlenstoffstrahlung behandelt werden, um die unterschiedlichen Strahlenqualitäten zu vergleichen?

-Zeigt das Zytostatikum Temozolomid eine Wirkung in GBM Slice-Kulturen?

-Lassen sich die Effekte der Behandlung anhand bekannter Marker für DNA-Schäden und Zelltod bestimmen?

Um die einzelnen Schritte der Experimente zu realisieren, wurden Kooperationen mit den Kliniken für Neurochirurgie der Universitätskliniken Leipzig und Mainz aufgebaut. Ebenso wurde die Zusammenarbeit mit der Klinik für Strahlentherapie des Universitätsklinikums Leipzig sowie der GSI Helmholtzzentrum für Schwerionenforschung begonnen.

Die Auswertung der Experimente wurde unter Anwendung immunhistochemischer sowie immunfluoreszierender Färbungen verschiedener Markerproteine bzw. morphologischer Charakteristika sowie Live-Imaging und semi-automatisierter Bildanalyse bearbeitet.

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4.1 Modeling radiation effects at the tissue level

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Regular Article

Modeling radiation effects at the tissue level

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Abstract. For the understanding of radiation action in humans, a synergistic approach of experiments and quantitative modeling of working hypotheses is necessary. A large set of experimental data at the single-cell level are available, and biophysical modeling of radiation action has so far mostly concentrated on the first phases of radiation interaction with the biomolecules, and later biochemical stages including DNA breakage, repair, and the formation of chromosomal aberrations. The models can be extended to cell killing and transformation, but so far still using a single-cell (clonal expansion) approach. On the contrary, new experimental evidence points to the microenvironment and the tissue level as a critical radiation target for late effects, and to epigenetic and signaling mechanisms as mediators of radiation damage. This higher structural level is generally ignored in modeling, owing to its complexity and to the lack of experimental data. In this paper we will highlight the requirements for specific experimental approaches targeting the tissue/microenvironment level and the most promising available experimental models.

1 Introduction

Modeling of biological radiation effects attracted the interest of physicists shortly after the first experimental evidence that ionizing radiation was a genotoxic agent [1]. In fact, unlike other mutagenic or clastogenic agents such as chemicals or viruses, ionizing radiation is very well characterized in terms of physical interaction with soft matter. Practically, the full initial energy deposition events can be described in terms of mathematical equations for ionizing radiation with unmatched precision in toxicology. With improvements in description of the biological targets, particular the chromatin in the cell nucleus [2], the mathematical models starting from track structure and energy deposition to cellular events are becoming more and more accurate.

The basic scheme is illustrated in Figure 1. Physical and chemical stages are fairly well characterized. The biochemical stage, i.e. the damage and repair of the DNA molecule, is still a central research issue in genetics [3]. Nevertheless, if the detailed enzyme kinetic is neglected, models based on repair-misrepair and pairwise interaction of DNA lesions are able to reproduce quite accurately the formation of DNA double-strand breaks [4], chromosome aberrations [5] and cell killing [6]. Much research in modeling is now concentrating on the chemical and biochemical stage to reproduce the actual processes at the cellular level [7].

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However, emerging evidence shows that ionizing radiation acts directly on the tissue microenvironment. Cells are obviously not isolated in the body, but closely interact with other cells of the same or different types, and this interaction determines the tissue functionality. The target of radiation effects is indeed not (only) the single cell (nucleus) but the whole tissue microenvironment. This affects the cell phenotype, tissue composition and the physical interactions and signaling between cells. These alterations in the microenvironment can contribute to carcinogenesis and alter the tissue response to anticancer therapy [8], yet there is a lack of modeling of radiation effects at the tissue level. This does not reflect an underestimation in the scientific community of the importance of the non-targeted effects in determining late consequences of radiation exposure [9], but its complexity and lack of basic experimental data.

2 Bystander effect

Challenging the paradigm (Fig. 1) of the cell nucleus as the only target of radiation action is the so-called bystander effect phenomenon – i.e. the observation that cells not hit by radiation, but in proximity to cells exposed to high- or low-LET radiation, can also be affected by the radiation exposure [10]. In vitro experiments have demonstrated apoptosis, mutations, micronuclei, and other cellular effects in bystander cells, even if target cells are only hit in the cytoplasm [11] and not in the nucleus. The effect

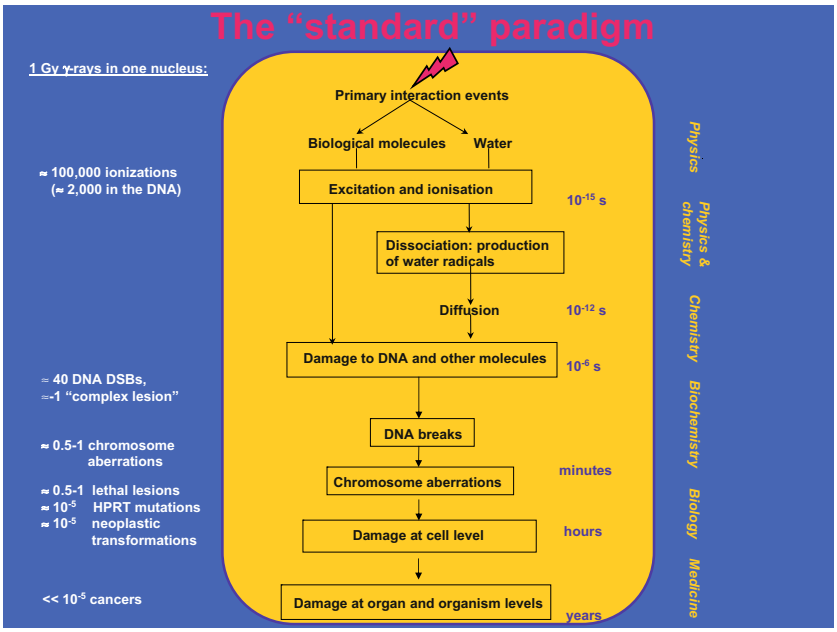

Fig. 1. (Color online) The classical paradigm of radiation action (graphics kindly provided by Dr. Andrea Ottolenghi, University of Pavia, Italy).

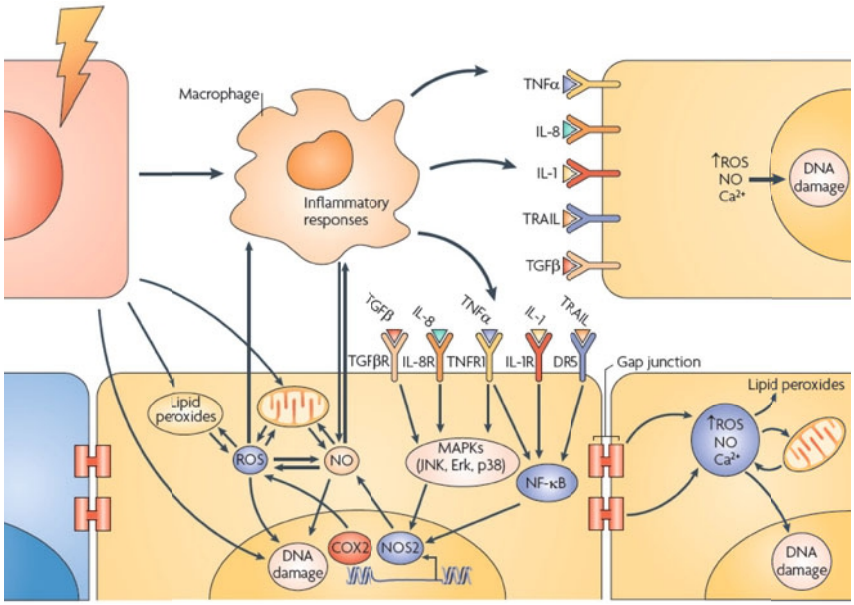

Fig. 2. (Color online) The two possible mechanisms responsible for the bystander effect. One involves direct cell-cell communication through gap junctions and the second release of cytokine signals into the ECM. COX2, cyclooxygenase 2; DR5, death receptor 5 (also known as TNFRSF10B); IL, interleukin; JNK, Jun N-terminal kinase; NO, nitric oxide; NOS2, NO synthase 2; ROS, reactive oxygen species; TGFβ, transforming growth factor-β; TGFβR, TGFβ receptor; TNFα, tumor necrosis factor-α; TRAIL, TNF-related apoptosis-inducing ligand. From [14], reprinted by permission from Macmillan Publishers Ltd.

has been confirmed by experiments in human skin tissues [12] and in mice [13]. Two mechanisms can be responsible for the bystander effect (Fig. 2): one involves direct cell-cell communication through gap junctions and the second release of cytokine signals into the extracellular matrix (ECM) [14]. The bystander effect is characterized by a non-linear response, i.e. by a rapid increase at low doses followed by saturation. Hence, at high doses targeted effects are obviously dominant, but the relative balance of targeted/non-targeted effect can change the shape of the dose-response curve in the low-dose region, of interest for radiation protection (Fig. 3) [15]. Interestingly, the bystander effect does not necessarily lead to an increase in the low-dose risk estimates: it can indeed act as a protective tissue mechanism, by eliminating the region where the damage occurred. Moreover, bystander effect can play a role in radiation oncology because part of the normal tissue is exposed to relatively low doses and can therefore trigger long-range tissue response, also known as "abscopal" effect in radiotherapy [16].

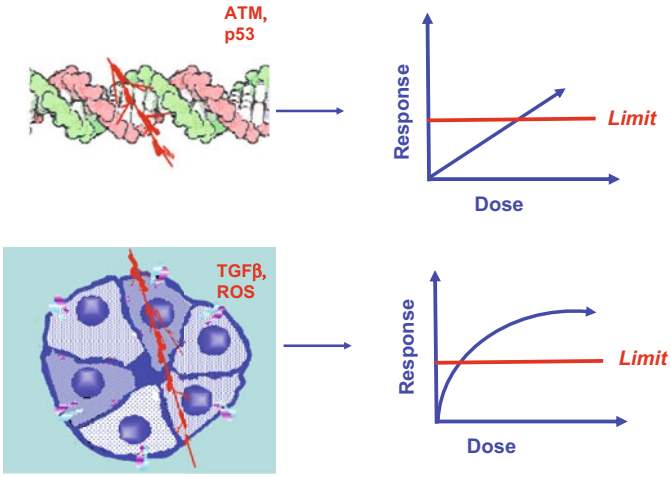

Fig. 3. (Color online) Impact of the mechanism of radiation action on the shape of the dose-response curve. A linear relationship is expected from DNA damage models, and the main enzymatic reactions involve DNA repair and cell-cycle checkpoint genes such as ATM and p53. Non-targeted effect should lead to non-linear responses, mediated by cytokines, TGFβ, and ROS (figure modified from Ref. [15]).

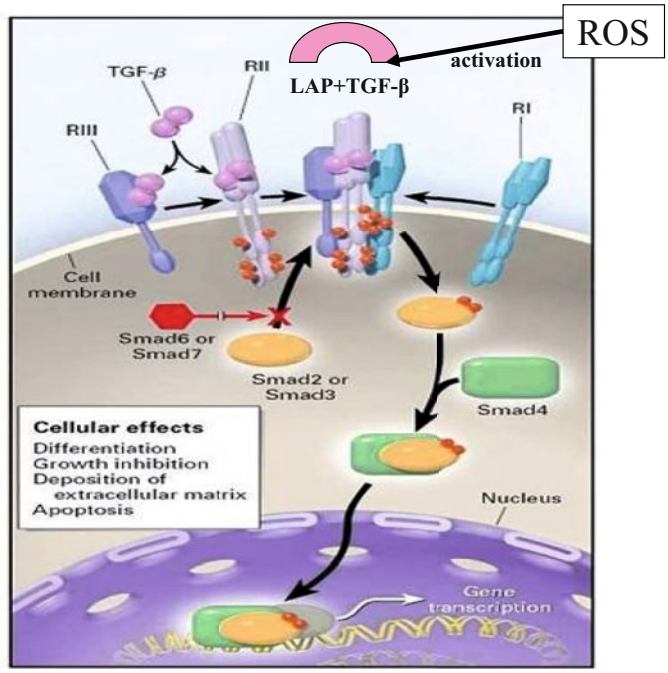

Fig. 4. (Color online) Pathway of TGFβ action. LAP = latency-associated peptide. Image modified from web collection.

3 Radiation and the microenvironment

When cells are exposed to radiation, DNA damage induces a stress response through activation or repression of distinct target proteins that primarily function to facilitate DNA repair and prevent the proliferation of damaged cells. TP53 and ATM play a pivotal role in this intracellular response, but many other proteins are involved. Similar to the stress response program within cells, radiation induces multicellular programs that orchestrate a response to damage at the tissue level [8]. Such programs are executed by soluble signals such as cytokines, growth factors and chemokines, which function on the parenchyma and stroma to modulate cell behaviors and phenotypes. Radiation can elicit an "activated" phenotype in some cells that promotes rapid, persistent stromal remodeling of the ECM. Remodeling of the ECM occurs through the induction of proteases and growth factors, and the chronic production of reactive oxygen species (ROS). Tissue responses to ionizing radiation are directed towards limiting damage, inducing repair and restoring tissue homeostasis. However, as with most tissue processes, this response can be disrupted by high doses of radiation, pre-existing conditions such as previous exposure, and the genetic features of the individual.

Oxidative stress is rapidly sensed by many proteins, and therefore ROS are universally considered the main agents affecting the microenvironment. Radiation is only one of the many ROS producing (at the chemical stage – see Fig. 1) agents, along with many chemicals and internal cellular processes. Oxidative stress can promote several pathological conditions, including those associated with ageing and cancer. It remains to be determined what levels of acute or chronic irradiation exceed the capacity of a given tissue to maintain homeostasis. Lessons learned from other processes that generate high levels of ROS, such as inflammation and ischemia/reperfusion, can be useful in identifying potential radiation-activated signals.

4 Cytokines

The response of the microenvironment to radiation and/or oxidative stress is mediated by cytokines, including epidermal and fibroblast growth factors, interleukines and pro-inflammatory cytokines. However, it is now clear that the transforming growth factor-β (TGFβ) is a key control molecule of tissue homeostasis, because it regulates both proliferation and apoptosis [17]. The pattern of TGFβ action is shown in Figure 4. This cytokine family exists in variant forms (TGFβ1, β2, and β3). The bioactive cytokine molecule is a dimer and it sends the signals by bringing together two pairs of receptor serine/threonine kinases, known as the type I and type II receptors. On binding TGFβ, the type II receptors phosphorylate and activate the type I receptors that then propagate the signal by phosphorylating the SMAD transcription factors. Once activated the receptor substrate SMADs (RSmads) shuttle to the nucleus and can bind the DNA. TGFβ exerts tumor-suppressive effects (regulating apoptosis, cytostasis, and differentiation) that cancer cells must elude for malignant evolution. Yet, paradoxically, TGFβ also modulates processes such as cell invasion, immune regulation, and microenvironment modification that cancer cells may exploit to their advantage in later stages of tumor growth [18]. Activation of TGFβ is an early and persistent event in tissues that have been exposed to ionizing

radiation [19], and as noted above it can have protective or damaging effects: for instance, it can protect the stem cell compartment in the intestine, but on the other hand it can promote invasion and metastasis in the epithelial tissue [8].

5 Experimental approach

Based on the short review presented above, it is clear that more experimental data are needed on non-targeted and tissue effects to model late consequences of radiation exposure. These data can be obtained in cells grown in vitro in 3D (spheroids), in animals, or in tissues removed from irradiated animals. A novel, promising approach is instead based on human slice culture preparations. The advantage of such slice cultures relies on the conservation of an organotypic environment, the easy treatment and observation by live-imaging microscopy, and the independency from genetic immortalization-strategies used to generate cell lines. It has been recently shown that entorhino-hippocampal organotypic brain slice cultures obtained from rats or mice can be kept in culture for up to several months and allow easy application of drugs and evaluation of their effects, e.g. using live-imaging [20]. With a similar technique, human tissues obtained from surgery can also be used to prepare slice cultures. No more than 30 min should pass between removal and slice preparation. It is possible to establish cultures from several different human tissues, including brain, tonsil, lymph nodes, and different kinds of solid tumors. While the survival of lymphoid tissues is presently restricted to about one week, tumors survived for extended periods of up to several weeks.

We are currently testing the radiation response of the tissue slices using heavy ions at GSI [21]. The use of heavy ions for these studies is particularly promising, because charged particles only traverse specific sections of the slice, which can then be visualized by immunostaining (Fig. 5). In addition, using low-energy ions (Fig. 6) it is possible to irradiate only one side of the tissue, and then to study the bystander response in the unirradiated side. The role of ROS and different cytokines, particularly TGFβ, can then be studied directly in live human tissues.

6 Modeling the radiation response of the microenvironment

Several attempts to model the non-targeted radiation effects can be found in the literature (e.g. [22]) but certainly the complex picture of interaction between damaged cells and the microenvironment (Fig. 7) begs for a more precise modeling based on more experimental data. This is particular important for heavy ions, which represent the main health risk in manned space exploration [23] and are used already in different centers for cancer therapy [24]. A schematic view of the interaction of heavy ion tracks and tissue is provided in Figure 8.

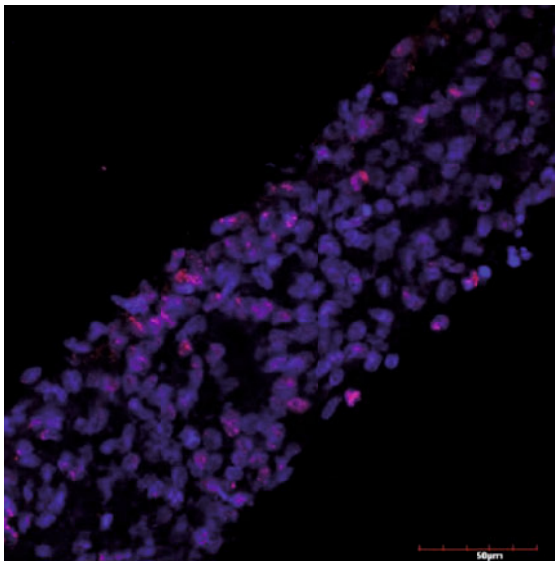

Fig. 5. (Color online) Images of rat hippocampus tissue slices irradiated with 11 MeV/n ^{12}C-ions at a fluence of 8×10^6 ions/cm^2 and fixed 1 h after exposure. Nuclei are stained in DAPI (blue) and DNA damage is visualized by γH2AX immunostaining (red). Clusters of cells carrying double-strand breakage (γH2AX nuclear foci) are evident in some areas of the tissue, those actually traversed by the carbon particles.

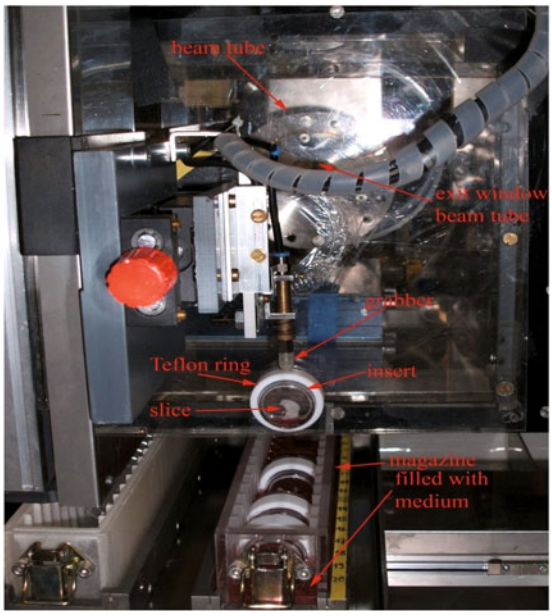

Fig. 6. (Color online) Irradiation setup for tissue slices installed at the low-energy heavy-ion accelerator (UNILAC) at GSI.

Modeling these effects is essential for understanding the relative biological effects (RBE) of heavy ions for late effects. For instance, a recent experiment at the Brookhaven National Laboratory accelerator has shown that the RBE of 1 GeV/n Fe-ions for cancer induction in mice is about 1 for leukemia and around 20 for hepatocellular carcinoma [25]. How can the RBE be so different?

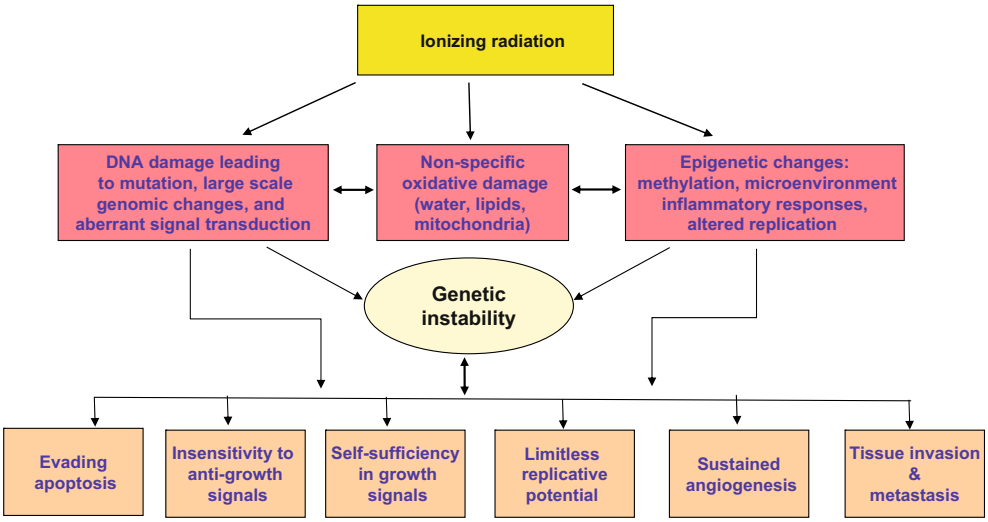

Fig. 7. (Color online) Schematic of the radiation action including the effect of the microenvironment (graphics courtesy of Dr. Frank Cucinotta, NASA Johnson Space Center, USA).

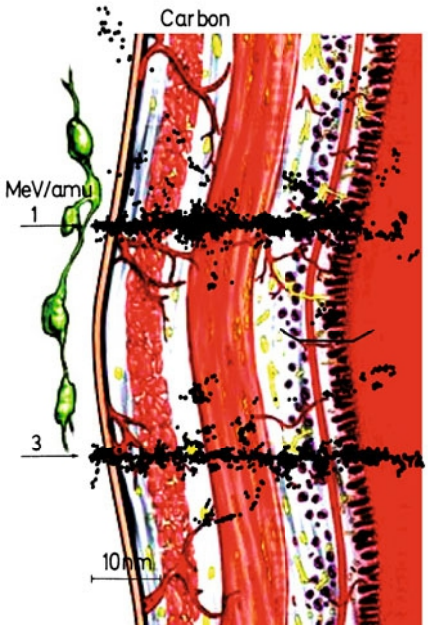

Fig. 8. (Color online) Schematic of the interaction of heavy ion tracks of different energies in human tissues. Tracks of carbon ions at two different energies (1 and 3 MeV/n) are superimposed to a schematic human tissue. The black dots represent the ionization events. Tracks and tissues are not in scale, in order to visualize the details of the track structure, which is in reality much smaller.

The reason is most likely due to the different nature of liquid and solid cancers. While leukemia is strongly related to specific chromosomal aberrations (in mouse, deletion of PU.1 gene in chromosome 2), solid cancers are associated to genomic instability (Fig. 7). It has been shown in normal human fibroblasts that heavy ions are a powerful inducer of delayed chromosomal instability [26]. Therefore, for liquid cancers radiation could act as an initiator, but for solid cancer as a promoter. Although heavy ions are more effective than X-rays in the induction of chromosomal rearrangements, most of the aberrations are lethal, and the RBE drops to about 1 in the surviving population [23]. On the other hand, heavy ions are very effective in the induction of inflammation [27], and as discussed above this process can lead to promotion of carcinogenesis. Hence, modeling the effects of charged particles in tissues can predict the RBE for different tumors. The physics of the interaction (Fig. 8) is very well known, and the production of ROS and their diffusion in the tissue can be accurately modeled with kinetics model. The activation of the TGFβ and other cytokines can also be modeled based on the experimental data that we hope to achieve in the near future with the ongoing experiments [21].

It has been suggested that fully stochastic cancer progression models incorporating malignant cell kinetics, dormancy (a phase in which tumors remain asymptomatic), escape from dormancy, and invasiveness, with radiation able to act directly on each phase, could predict the radiation risk and RBE [28]. It is unclear though whether a population dynamics approach for a diverse somatic cell population undergoing mutations or other alterations would be sufficient to model the tissues, without taking into account a higher organizational level. An alternative theoretical approach to individual cell-based models are indeed the continuum models [29], where the tissue phase can be treated using a global-scale theoretical approach, for instance the theory of viscous liquids [30]. These mathematical models may prove to be powerful in modeling radiation response of the microenvironment.

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4.2 Tissue slice cultures from humans or rodents: a new tool to evaluate biological effects of heavy ions

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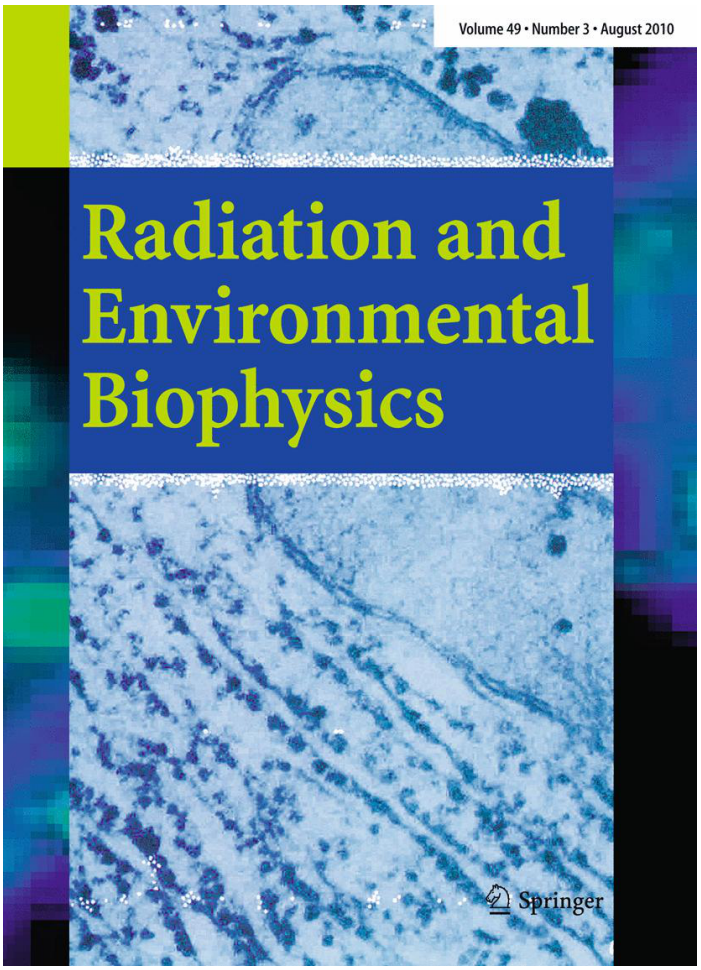

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ORIGINAL PAPER

Tissue slice cultures from humans or rodents: a new tool to evaluate biological effects of heavy ions

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Abstract The aim of this interdisciplinary project is to establish slice culture preparations from rodents and humans as a new model system for studying effects of X-rays and heavy ions within normal and tumor tissues. The advantage of such slice cultures relies on the conservation of an organotypic environment, the easy treatment and observation by live-imaging microscopy, and the independence from genetic immortalization strategies used to generate cell lines. Rat brains as well as human tumors were cut into 300-μm-thick sections and cultivated in an incubator in a humidified atmosphere at 37°C. This is realized by a membrane-based culture system with a liquid–air interface. With this system, it is possible to keep rodent slices viable for several months. Human brain tumor slices remained vital for at least 21 days. Slices were irradiated with X-rays at the radiation facility of the University Hospital in Frankfurt/Main at doses up to 40 Gy. Heavy ion irradiations were performed at GSI (Darmstadt) with different ions, energies, and doses. The irradiated slices were analyzed by 3D-confocal microscopy following immunostaining for DNA damage, microglia, and proliferation markers. The phosphorylated histone γH2AX proved to be suitable for the detection of ion traversals in this system.

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Introduction

The "London tragedy" in March 2006, when six healthy volunteers experienced toxic shock syndromes after receiving the novel drug TGN1412 (Kenter and Cohen 2006), emphasized how far away current biological test systems can be from the human situation. The drug was approved in England and Germany after extensive trials including injections into monkeys where no adverse effects had been observed. Since the incident, various committees of experts have been proposing the need for innovative test systems using human tissues (Dowsing and Kendall 2007). However, research on future therapies often ignores these caveats and remains restricted to experiments with primary cell cultures, often from rodents, or immortalized cell lines. Also, it is rarely appreciated that e.g. extracellular matrix molecules and local cytokine profiles strongly impact on cell differentiation (Schaefer et al. 2005). Therefore, isolating cells from their organotypic environment causes

de-differentiation, which certainly has massive impacts on the molecular patterns determining survival and apoptosis. Thus, besides the often underestimated species differences, cell culture studies involve various pitfalls that deserve to be acknowledged when data raised in such systems are interpreted in the context of human diseases and their therapies. Here, we describe (human) tissue slice culture as an alternative experimental system, which allows avoiding several sources of artifacts and misinterpretations, but offers several advantages over animal experiments such as the option to work with human tissues, the open access for reproducible treatment with drugs or exposure to radiation, and the possibility to perform live-imaging to visualize cellular events such as migration or cell death. Also, in continuation of successfully applied 3D culture systems (Belyakov et al. 2005; Roig et al. 2009; Su et al. 2009; Sowa et al. 2010), slice cultures display the tissues' natural three-dimensional cytoarchitecture resembling the in vivo situation. This represents a strong advantage of slices because a de-differentiation does not occur and the extracellular matrix remains intact.

We have previously established entorhino-hippocampal organotypic brain slice cultures obtained from rats and mice (Dehghani et al. 2003; Eyupoglu et al. 2004; Hailer et al. 2001; Kluge et al. 1998; Kreutz et al. 2007). Such slice preparations can be kept in culture for up to several months and allow easy application of drugs and evaluation of their effects, e.g. using live-imaging (Aktas et al. 2005). The advantage of the entorhino-hippocampal system relies on the 3-D organization of this brain area: the major fiber tract into the hippocampus, the perforant path, derives from entorhinal neurons and is oriented perpendicularly to the longitudinal axis of the C-shaped hippocampus. The perforant path can be preserved within the slice preparation with appropriate cutting as we have shown using tracer injections and electron microscopy (Kluge et al. 1998). Live-imaging (two-photon) microscopy is a useful tool for visualizing the slices. It is possible to follow the migratory pathways of T cells through living tissues slice in order to study their impact on neuronal calcium influx and/or survival. To this end, calcium-sensitive dyes such as FURA can be added to the cultures. A recent study by Nitsch et al. (2004) applying this technique revealed a previously unknown rapid calcium influx into neurons after contact with T lymphocytes.

The early activation of microglia throughout the slice, which is driven mainly by the acute anterograde and retrograde degeneration of axons, ceases within a few days. Subsequently, microglia, which are regarded as the early sentinels (Kreutzberg 1996) of the brain, exhibit a ramified, previously called "resting state" indicating the absence of further degeneration (Hailer et al. 1996). Neuronal cell death induced by stimuli of interest can be evaluated and quantified by using propidium iodide in histological preparations of the fixed slice (Ullrich et al. 2001). Cryosections can be cut from slices in which most protocols of immunocytochemistry are applicable allowing in-depth histopathological analysis (light, confocal, electron microscopy) at the end of a given experiment. Thus, besides following the effects of certain stimuli over time in living preparations using live-imaging or measurements of certain parameters, such as cytokines in supernatants, the final outcome can be analyzed/quantified by virtually all means of state-of-the art-morphology.

Within ethical restrictions, tissues obtained from surgery can also be used to prepare slice cultures when being rapidly processed. Using this approach with tissues from epilepsy surgery, we recently detected an important species difference: the death ligand TRAIL, which was not toxic in mice (Walczak et al. 1999), induced vast degeneration of human neurons, oligodendrocytes, and astrocytes (Nitsch et al. 2000). These findings encouraged us to extend slice preparations to additional human tissues including tonsils, lymph nodes, cancer metastasis, and brain tumors. While the survival of lymphoid tissues is presently restricted to about 1 week, tumors survived for extended periods of up to several weeks. One tumor which is of particular interest due to the current lack of effective treatment is the glioblastoma multiforme (GBM). It is possible to dissect extended tissue blocks thereby allowing preparation of sufficient amounts of slices from one patient to perform comparative studies.

Aimed at systematically studying the effects of heavy ions versus X-ray in slice preparations, we have started to test the influence of irradiation. In this paper, we describe the establishment of the experimental procedure and first results.

Materials and methods

Preparation of organotypic entorhino-hippocampal and glioblastoma slices

Details have been published previously (Eyüpoglu et al. 2003; Kluge et al. 1998; Kreutz et al. 2009). Briefly, p6 rats were decapitated, and the brains were removed from the skull under aseptic conditions. Brains were rinsed in ice-cold Hanks balanced salt solution (HBSS) and fixed into a vibratome. Serial sections of 300 μm were cut from which the entorhino-hippocampal formation was dissected. These sections were transferred onto membrane inserts seated into media-filled six-well plates where the typical morphological organization of the hippocampus was observed by live microscopy (Fig. 1). For glioblastoma slices, tissues freshly obtained from surgery were cut in 300 μm

slices using a vibratome. Treatment of the sections was as described previously. Slices were kept in culture for 7 days before they were exposed to radiation.

Exposure of slices to heavy ions

Heavy ion exposure was performed at the low-energy (UNILAC) and high-energy (SIS) facilities at GSI. Slices were irradiated with fluences ranging from 0.5×10^6 to 8×10^7 p/cm^2. For irradiation at UNILAC, culture inserts were fixed upright in custom-made attachments in sample holders filled with medium. The sample holders were fitted in the irradiation chamber and inserts were then automatically raised by a grabber into the horizontal beam line (Fig. 2). This procedure did not take more than 15 min and control slices were included in each sample holder. Dosimetry was performed using ionization chambers and etching of CR39 nuclear track detectors for calibration (Kraft et al. 1980). Irradiation at SIS, where the energy can reach 2 GeV/n, was performed in Cave A using a remote-controlled positioning system. In pioneering experiments, slices from human GBM (Fig. 3), which could be maintained in culture for at least 21 days, were exposed to carbon ions. Heavy ion effects on these tissues will be explored in future experiments. Data of exposed samples are shown after Carbon (9.8 meV/u on target, LET 170 meV/u) and Xe ion (4.5 meV/u, LET 8800 keV/µm) irradiation. Carbon ions of this energy penetrate 417 µm into tissue, whereas Xenon ions reach 78 µm only.

X-ray irradiation

Parallel to the heavy ion irradiations, X-ray experiments were performed at the Clinics for Radiation Therapy and Oncology at the University Hospital in Frankfurt. Slices were irradiated in 6-well plates at the patient irradiation site. X-rays were generated by a 6MV LINAC (Elekta). Samples were irradiated at 1, 2, and 4 Gy, in accordance with therapeutic doses. Due to the high radio-resistance of brain tissue, a very high dose of 40 Gy was chosen in order to test whether histological techniques are suitable to detect tissue degeneration.

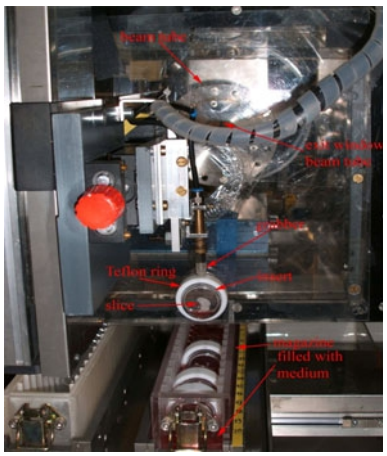

Fig. 2 Irradiation setup at GSI. The slices on culture inserts were fixed in special rings and kept in a sample holder filled with medium. For irradiation, they were raised into the beam line with a grabber

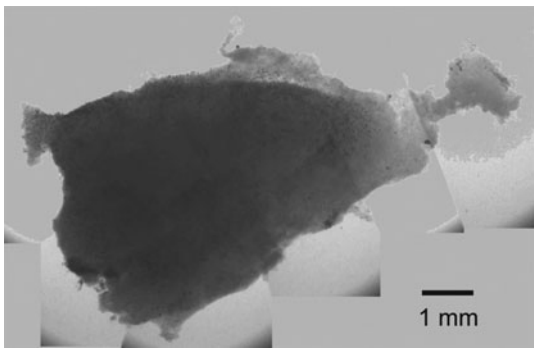

Fig. 3 Slice culture of a human glioblastoma multiforme. These tumors are often removed in pieces of several centimeters thus providing sufficient tissue for diagnostic and experiments. Due to the inter-individual heterogeneity, it is important to compare the effects of X-ray and heavy ions in slices of the same origin (i.e. patient). Note the total length of 7 mm

Immunocytochemistry

After irradiation, the samples were fixed with 4% paraformaldehyde at various time-points ranging from 1 h up to 10 days. The slices were then cryosectioned (20 µm) vertically in order to preserve the complete lateral profile of the irradiated tissue. Standard protocols (Kluge et al. 1998)

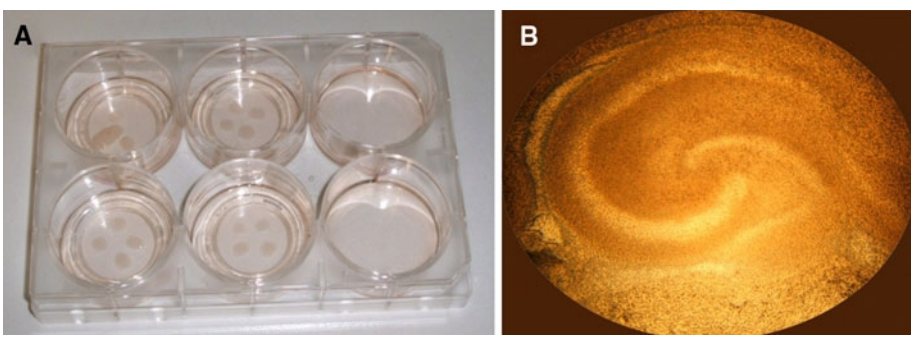

Fig. 1 Entorhino-hippocampal slice preparation. **a** Slice cultures on membranes in six-well plates. **b** A microphotograph of a living slice. The two "U"s of the hippocampus (cornu amonis and the granule cell layer of the dentate gyrus) are visible due to lower density compared to white matter areas

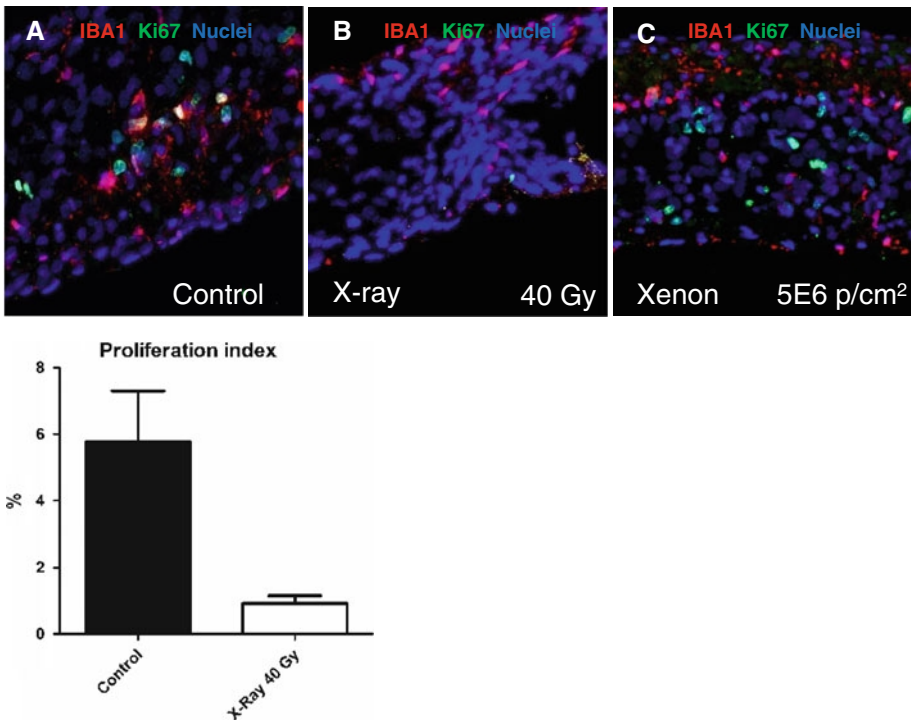

Fig. 4 Effects of X-ray and ^{136}Xenon (4.5 meV/u on (microglial) proliferation in normal rat brain slices. In accordance with the expectation, irradiation with 40 Gy blocked the normal proliferation (detected by Ki67-staining) seen in control slices (Fig. 4a–b). Respective counts are shown in the *panel* below (*Bars*: SD, $p < 0{,}0064$ determined by *t*-test). In Xenon-treated slices, proliferating cells were still visible. This is not astonishing, because the energy was not deposited homogenously throughout the slice as it is the case for X-ray

for (double/triple) immunocytochemistry were applied to detect e.g. nuclear DNA damage and repair, proliferation, and microglial activation. Primary antibodies were directed against IBA1 (WAKO; 1:200), phospho-histone H2AX (Ser139) (Millipore; 1:100), and Ki67 (BD Bioscience; 1:100). Secondary Antibodies were goat anti-mouse or anti-rabbit conjugated with Alexa dyes (Invitrogen; 1:250). Sections were analyzed using an Olympus FV-1000 confocal microscope. For quantitative analysis of proliferation, the total number of Ki67-positive cells within three-dimensionally reconstructed areas of 20 µm depth at 400× magnification was counted and related to the number of nuclei from 9 z-stacks per group. This ratio was analyzed statistically by *t*-test.

Results

Microglia are known to proliferate in response to various pathologic stimuli (Hailer et al. 1999) as they occur in slice preparations (Hailer et al. 1996). In line with this notion, proliferating microglia were visible in triple-stained sections in which nuclei, microglial cells and the proliferation marker Ki67 were labeled simultaneously (Fig. 4a, control). As anticipated, proliferation ceased at 96 h in slices exposed to X-ray at a dose of 40 Gy (Fig. 4b). In Xenon-treated slices, proliferating cells were still visible, but it is likely that due to the short range of the ions these cells were not hit. Therefore, we sought for a method to identify areas of energy deposition within individual slices. A likely candidate was phosphorylated histone H2AX (γH2AX), an established marker for ion-induced DNA double-strand breaks (Jakob et al. 2003). In fact, when irradiated at a fluence of 8×10^6 p/cm^2 of ^{12}Carbon particles at the UNILAC facility, single "particle tracks", i.e. parallel streaks of punctuate staining across nuclei, were visualized by γH2AX -staining 1 h after irradiation (Fig. 5a–d). This is a clear indication of ion traversal–induced DNA damage. At a higher fluence (8×10^7 p/cm^2), DNA damage was clearly enhanced so that complete nuclei appeared to be filled with the phosphorylated protein (Fig. 5f). While IBA-1-positive microglial cells exhibited amoeboid morphologies at the surface of the slice, they showed tiny ramification under the surface indicating an immunologically un-stimulated state despite of exposure to carbon ions (Fig. 5c). Importantly, γH2AX was almost absent in untreated control slices; only single cells, most likely proliferating microglia, were found (Fig. 5e) indicating γH2AX as a suitable marker to identify areas of ion entry and/or traversal.

Although rat and human slices were temporarily removed from their media during exposure to heavy ions, subsequent degeneration was not evident. Rat and GBM slices survived for at least 10 days after exposure allowing a wide range of biological reactions to be studied.

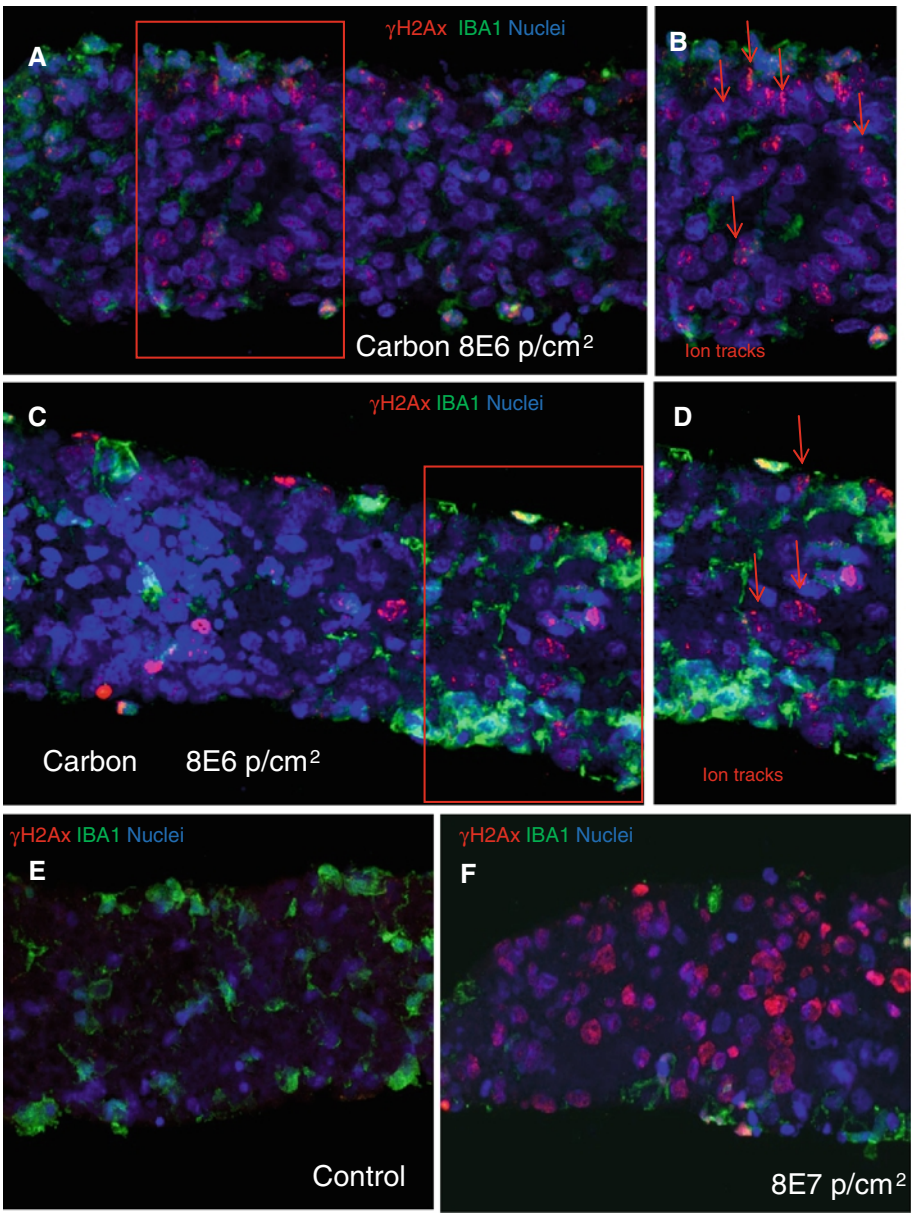

Fig. 5 Effects of X-ray versus ^{12} Carbon (9.8 meV/u) on DNA damage. When irradiated with a fluence of 8×10^6 p/cm^2, particle tracks could be visualized by staining for phosphorylated γH2AX 1 h after irradiation (**a**–**d**). At a higher fluence (8×10^7 p/cm^2), DNA damage was clearly enhanced so that complete nuclei appeared to be filled with the phosphorylated protein (**f**), whereas DNA damage or γH2AX-staining respectively was absent in untreated controls (**e**)

Conclusions

Current experimental strategies for the development of innovative therapies using cell lines often suffer from artifacts due to immortalization, de-differentiation, and species differences. Exploiting the effects of potential therapies in (human) tissue slice cultures circumvents these drawbacks by keeping cells within their organotypic environment. Starting to search for "human" test systems for heavy ion irradiation, our data demonstrate that (i) slice cultures represent a suitable model to investigate the effects of X-ray and heavy ion irradiation, and (ii) γH2AX is a suitable marker to detect areas of ion entry and/or traversal in slice preparations. Current studies address effects of heavy ions on cell survival and DNA repair in different cell populations (microglia, oligodendrocytes, astrocytes, and neurons) in the normal rat brain and human GBM.

Acknowledgments This project has been funded by the Kassel-Stiftung, the Messer-Stiftung, the Beilstein-Stiftung and the Dr. Senckenbergische-Stiftung. We are truly grateful for the start-up funding and the since then continuous support of these Frankfurt foundations. Aspects of our work with slice cultures are now financed by the Bundesministerium für Bildung und Forschung (BMBF to I.B.) and the European Space Association (ESA to I.B. and G. T.-S.).

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4.3 Irradiation of human tumor tissue cultures: optimizing ion radiation therapy

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Editorial
Future Oncology

Irradiation of human tumor tissue cultures: optimizing ion radiation therapy


"...we are acquiring useful and previously unknown information regarding heavy ion effects on (tumor) tissues."

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Irradiation provides a key therapy that is highly beneficial for many tumors. However, finding proper doses and treatment regimes is mainly based on clinical studies and therefore, it takes years or even decades to develop effective protocols. We have established a system to irradiate slice cultures of human tumors with the ultimate goal of testing and studying the biological effects of irradiation with photons and heavy ions on cells in their organotypic environment. In this setting, irradiation-induced modes of cell death and mechanisms of resistance can be investigated using the complete spectrum of state-of-the-art morphology, pharmacology and molecular biology. Moreover, it is possible to systematically address the effects of fractionating and thus to determine the most effective regimes. This can also take into account circadian rhythms of tumors, an issue that is rarely addressed in the field. Eventually, it may be possible to use such cultures obtained from tumor surgery to define the most effective therapy for the individual patient. Since the organotypic environment strongly impacts on cellular differentiation (e.g., by signaling of matrix molecules [1,2]), similar approaches using cultures of isolated cancer cells may have failed in the past. With regard to heavy-ion therapy, fundamental questions on their biological effects in tissues are still to be investigated, and slice cultures provide an ideal tool to address them.

The London tragedy, in which six volunteers experienced severe toxic shock symptoms upon treatment with the novel drug TGN1412, impressively demonstrated that prediction of the response to drugs cannot be safely based on animal experiments [3,4]. Since then, various European and transatlantic committees have called for development of human test systems in order to avoid species differences [5]. We had previously demonstrated that the death ligand, TNF-related apoptosis-inducing ligand, which was well tolerated in mice [6], exhibited strong proapoptotic effects in human brain tissue slices obtained from epilepsy surgery [7]. Using slice cultures from rodents, we have developed protocols for histological analysis, including confocal and electron microscopy, in order to study basic principles of degeneration and cellular differentiation in their organotypic environment [8–11]. We have also employed this approach to investigate the action of lymphoyctes *in situ* [12], and we are currently using slice cultures of tonsils for the development of a human model of the immune system and its responses to novel drugs.

"We have established a system to irradiate slice cultures of human tumors with the ultimate goal of testing and studying the biological effects of irradiation with photons and heavy ions on cells in their organotypic environment."

The basic principle of slice cultivation is simple: tissues are cut in 300 µm sections and transferred on membrane culture inserts that are placed onto the surface of a medium in six-well plates [13,14]. Cutting can be performed with tissue choppers, but we found that vibratomes yield better results with regard to tissue preservation (Figure 1). At the end of the experiment, tissues can be fixed for histological analysis. All morphological standard techniques work well with these tissues. For example, it is possible to perform fluorescent stainings in order to identify cells exhibiting radiation-induced DNA double-strand breaks (Figure 2). Cell death can be visualized using propidium iodide [10].

Keywords
■ glioblastoma ■ heavy ion irradiation ■ human test system ■ replacement of animal experiments ■ species difference ■ tumor slice culture

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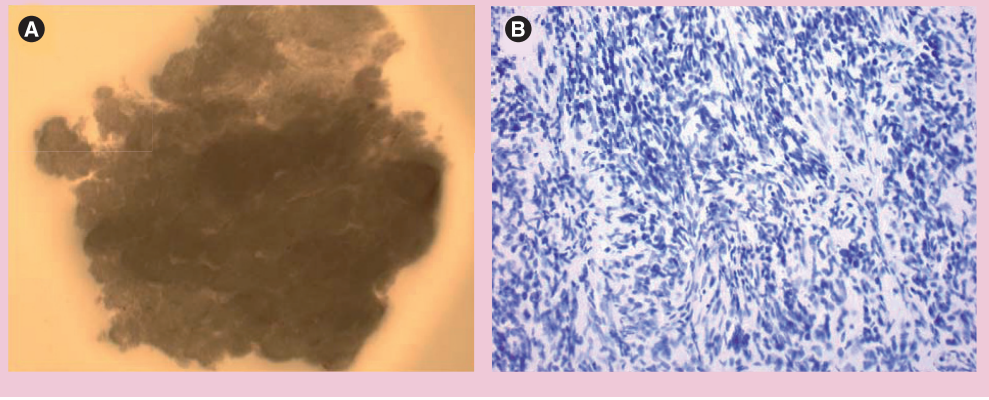

Figure 1. Human tumor slice cultures preserve tissue characteristics over a time of at least 2 weeks. Human tumor slice culture obtained from **(A)** the brain in culture and **(B)** after fixation and standard histological preparation (hematoxylin–eosin and paraffin).
(A) Magnification: 40×. **(B)** Magnification: 100×.

While live imaging in slices is possible, it is currently difficult to completely transfect sliced tissues with respective green fluorescent protein constructs, which would allow for the identification of a particular cell type. Besides morphological evaluation, the open system allows for controlled and reproducible application of drugs and acquisition of supernatants (e.g., to measure certain cytokines). Of course, it is easy to perform molecular biological tests of homogenized slices at the end of the experiment.

For irradiation, slices can be kept in six-well plates and irradiated under different conditions using conventional photons as a control, or heavy ions with low and high fluence, as well as an extended Bragg peak (using carbon ions) [15]. There is certainly room for improvement; we have only begun to analyze histological sections of human glioblastoma slices exposed to photons and carbon ions. Routine analysis currently involves the counting of cell deaths, DNA damage and proliferation by hand. Automated counting of serial sections would certainly be helpful for speeding up the process, and we are working on employing tools to this end. At the same time, we are acquiring useful and previously unknown information regarding heavy ion effects on (tumor) tissues.

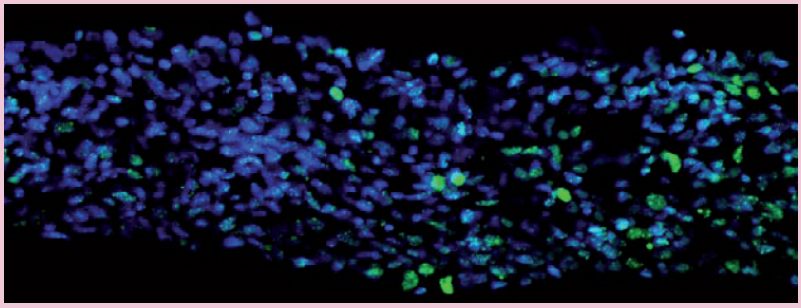

Figure 2. DNA double-strand breaks in a human glioblastoma slice culture (nuclei in blue) after carbon irradiation visualized by γH2AX staining (green).
Cryosection: 16 µm; magnification: 200×.

> "The London tragedy, in which six volunteers experienced severe toxic shock symptoms upon treatment with the novel drug TGN1412, impressively demonstrated that prediction of the response to drugs cannot be safely based on animal experiments."

It is encouraging for us to see that others also feel that the enthusiasm over novel genetic tools in mice has caused a long period of neglecting studies in human tissues [16]. In truth, we believe that little can be learned about the human situation from studies using immortalized 'cancer' cell lines injected into immunodeficient mice. Although we may be a (very) long way from developing individualized therapies based on assays using the patient's own tumor tissue, we are not wasting our time; rather, we will learn a lot about the biology of 'real' tumors and the effects of irradiation therein.

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4.4 Organotypic slice cultures of human glioblastoma reveal different susceptibility to treatments

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Organotypic slice cultures of human glioblastoma reveal different susceptibilities to treatments

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Background. Glioblastoma multiforme is the most common lethal brain tumor in human adults, with no major therapeutic breakthroughs in recent decades. Research is based mostly on human tumor cell lines deprived of their organotypic environment or inserted into immune-deficient animals required for graft survival. Here, we describe how glioblastoma specimens obtained from surgical biopsy material can be sectioned and transferred into cultures within minutes.

Methods. Slices were kept in 6-well plates, allowing direct observation, application of temozolomide, and irradiation. At the end of experiments, slice cultures were processed for histological analysis including hematoxylin-eosin staining, detection of proliferation (Ki67), apoptosis/cell death (cleaved caspase 3, propidium iodide), DNA double-strand breaks (γH2AX), and neural subpopulations. First clinical trials employed irradiation with the heavy ion carbon for the treatment of glioblastoma patients, but the biological effects and most effective dose regimens remain to be established. Therefore, we developed an approach to expose glioblastoma slice cultures to ^{12}C and X-rays.

Results. We found preservation of the individual histopathology over at least 16 days. Treatments resulted in activation of caspase 3, inhibition of proliferation, and cell loss. Irradiation induced γH2AX. In line with clinical observations, individual tumors differed significantly in their susceptibility to temozolomide (0.4%–2.5% apoptosis and 1%–15% cell loss).

Conclusion. Glioblastoma multiforme slice cultures provide a unique tool to explore susceptibility of individual tumors for specific therapies including heavy ions, thus potentially allowing more personalized treatments plus exploration of mechanisms of (and strategies to overcome) tumor resistance.

Keywords: glioblastoma multiforme, organotypic slice culture, human test system, heavy ions.

Glioblastoma multiforme (GBM) is among the most common lethal tumors, with patients having an average life expectancy of <18 months after diagnosis. While substantial progress has been made with regard to understanding tumor pathogenesis,[1,2,3,4–7] expansion,[8] bystander damage,[9] migration,[10] altered protein expression, and resistance to cell death,[11] none of this information significantly affects life expectancy or quality of life.

Current research often employs immortalized cell lines that are studied in co-culture or after transplantation in immunodeficient mice. While such approaches

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certainly help identify basic principles of tumor biology and immunology, it is difficult to directly translate observations to the human disease and to develop accurate or even individualized therapeutic strategies based on such experiments. We have previously used organotypic entorhinohippocampal slice cultures in which all neural subpopulations of cells and the basic interneuronal connections are maintained.[12–16] These organotypic slice cultures include several advantages, such as (i) the presence of the organotypic matrix, which is increasingly appreciated as providing crucial signaling for site-specific cellular differentiation[17–20]; (ii) open access allowing treatment and direct observation over extended periods of time; and (iii) collection of supernatants for analysis over time. Using brain slice cultures obtained from epilepsy surgery, we have shown that normal human brain cells, in contrast to the murine system, are susceptible to lysis by tumor necrosis factor–related apoptosis-inducing ligand.[21,22] This exemplified the need for the development of human test systems for the evaluation of toxicity and preclinical research, an issue that gained worldwide attention after the "London tragedy," in which 6 volunteers experienced toxic shocks and cytokine storms in response to an anti-CD28 antibody that had been previously well tolerated by rodents and monkeys.[23,24,25] Over the last few years, the use of human slice cultures of tumors in research has begun to emerge in the literature,[26,27] underlining an emerging awareness of species differences.

One possible novel approach to treating GBM could be irradiation with high-energy carbon ions. Heavy ion (HI) therapy was developed over 50 years ago at the Lawrence Berkeley Laboratory in the United States and is currently in operation in several centers in Europe and Asia.[28] The rationale of using particles heavier than protons for therapy lies in their special radiobiological characteristics, especially in their ability to overcome radioresistance.[29] This high relative biological effectiveness makes them attractive for use against tumors resistant to conventional therapy, such as GBM. Clinical results support the rationale of the therapy, and a clinical trial on glioblastoma is currently under way in Heidelberg.[30]

Here, we tested the survival and suitability of slice cultures derived from GBM as a test system for current and future therapeutic strategies, based on our previous findings.[31,32] Tumor tissues obtained directly from neurosurgical operations were immediately transported to the laboratory in media, cut into 350-μm sections, and kept on membranes in 6-well plates for up to 4 weeks. We exposed GBM slice cultures to temozolomide (TMZ), X-rays, and irradiation with the HI carbon (^{12}C) and monitored the effect on proliferation, cell death, and DNA double-strand breaks (DSBs). Our data showed that therapeutic effects can be mimicked well in such slice preparations, which therefore are potentially suitable as an experimental model to better understand mechanisms of tumor resistance, as well as a test system for novel therapies and susceptibility assays for personalized treatment.

Table 1. Glioblastoma samples used for slice culture experiments in this study

Sample	Experiment	Data Shown In
03082010	Long-term culture	Fig. 1
12082010	Long-term culture	Fig. 1
25072012	Long-term culture and X-irradiation	Figs. 1 and 3
12122011	TMZ treatment	Figs. 2, 6, and 8
10092010	Live imaging and TMZ treatment	Fig. 5
16092010	Live imaging and TMZ treatment	Fig. 5
03012011	Live imaging and TMZ treatment	Fig. 5
05042011	Carbon ion irradiation and TMZ treatment	Figs. 3, 8, 4, and 6
11102011	Carbon ion irradiation and TMZ treatment	Figs. 7 and 8
24062011	Carbon ion irradiation and TMZ treatment	Figs. 7 and 8
11052012	TMZ treatment and X-irradiation	Fig. 8
15082012	TMZ treatment and X-irradiation	Fig. 8

All patients had a diagnosis of World Health Organization grade IV GBM.

Materials and Methods

Tissue Slice Preparation

All patients provided written informed consent according to German law as confirmed by the local committees (144–2008 and 837.211.12-8312-F). Glioblastoma tissue not required for neuropathological diagnostic procedures was obtained after surgical resection at the Department of Neurosurgery (Leipzig or Mainz). An overview of the samples used in this study is given in Table 1. The tissue was transported to the laboratory in minimal essential medium (MEM; Invitrogen). Slice cultures were prepared using a vibratome (Leica VT 1000) or a tissue chopper (McIlwain TC752) at a thickness of 350 μm under sterile conditions. Before preparations, a standard razor blade was wiped with ethanol to remove any oil and then sterilized by autoclaving. Additionally, a normal glass pipette and a pipette with the fine tip broken off were autoclaved. If needed, biopsy specimens were cut into appropriate-size pieces first to obtain evenly shaped slices of $\sim 5 \times 5$ mm. When the tissue chopper was used, the tissue was put on a stack of sterile filter membranes, cut, and then transferred carefully into ice-cold MEM by forceps. In most of the preparations, the slices stuck together after cutting, so they were separated under a stereomicroscope with 2 scalpels without cutting into the tissue. Slices were then transferred by the glass pipette with the wide opening onto membrane culture inserts (Millipore) in 6-well plates at a maximum of 3–4 slices per insert depending on size. The cultivation medium consisted of MEM (Gibco), 25% Hank's Balanced Salt Solution (with Ca and Mg; Gibco), 25% N-hydroxysuccinimide (Gibco), 1% L-glutamine (Braun), 1% glucose (stock solution 45%, final concentration 0.45%; Braun), and 1% penicillin/streptomycin (Sigma). Slices were cultivated on a

liquid/air interface in a humidified incubator at 37°C and 5% CO_2. Medium was changed 3 times a week. After time points ranging from 1 h to 4 weeks, slices were fixed in 4% paraformaldehyde and processed for paraffin embedding. Paraffin sections (8 μm) were cut and stained with hematoxylin and eosin (H&E) for histology. Histology of these sections was compared with the diagnostic histopathology of the same tumor to detect tissue culture–induced changes. For immunocytochemistry, sections were dewaxed in xylene, rehydrated in a decreasing alcohol series, and (if needed) pretreated for antibody staining with citrate buffer (pH 6) in a microwave. Then, sections were washed in phosphate buffered saline (PBS), permeabilized with 1.5% Triton/PBS for 10 min, blocked with 10% normal goat serum in 1.5% Triton/PBS for 1 h, and incubated overnight at 4°C with primary antibodies against Ki67 (rabbit, 1:100; DCS), cleaved caspase 3 (rabbit, 1:400; Cell Signaling), glial fibrillary acidic protein (GFAP; Dako, rabbit, 1:600; Dako), nestin (rabbit, 1:600; Chemicon), vimentin (mouse, 1:100; Dako), neurofilament (mouse, 1:100; Dako), or γH2AX (mouse, 1:100; Millipore). Visualization was achieved by incubation either with appropriate fluorescent-labeled secondary antibodies (goat anti-mouse or anti-rabbit Alexa 488) or with biotinylated anti-rabbit immunoglobulin G followed by streptavidin-conjugated horseradish peroxidase and developing by adding diaminobenzidine for the color reaction. For fluorescent staining, photographs were taken using an Olympus BX51 fluorescent microscope or a Zeiss LSM 510 confocal microscope. In addition to the green fluorescent channel, the red channel was included because some of the samples exhibited a strong autofluorescence, which is normal in the nonjuvenile human brain. Only cells devoid of red fluorescence were used for further analysis. For H&E and diaminobenzidine staining, a Zeiss Axioplan 2 microscope was used.

Analysis of Proliferation in Slices

Paraffin sections (8 μm) of slices were dewaxed as previously described here, and proliferating cells were stained with the Ki67 antibody. Nuclei were counterstained with Hoechst 33342. Then, at least 12 images were acquired of 4–6 different sections per group and manually analyzed using ImageJ and the PlugIn CellCounter. The percentage of Ki67-positive cells in relation to the total cell number was referred to as the proliferation index. Statistics were performed using GraphPad Prism 5 (Student's *t*-test).

Treatment of Glioblastoma Tissue Slices With Temozolomide

Glioblastoma tissue slices maintained on cell culture inserts were incubated with TMZ (dissolved in dimethyl sulfoxide) at a final concentration of 50 or 200 μM of the compound. Control slices were incubated with the corresponding amount of dimethyl sulfoxide (0.2% v/v). After 72 h, tissue slices were removed from the membranes and incubated in 500 μL of medium and 10 μg/mL of Hoechst 33342 (Sigma) at 37°C and 5% CO_2 for 1 h to allow for nuclear staining. The tissues were then transferred into 1 mL PBS containing 2 μg/mL propidium iodide (PI; Invitrogen) and gently fixated between 2 glass coverslips.

For confocal imaging and quantification of viable and dead cells within the tissues, an LSM-510 META inverted laser scanning microscope and a 20×/0.8 Plan-Apochromat objective (Carl Zeiss MicroImaging) were used. Hoechst 33342 was excited at 364 nm. PI was excited with the 543-nm laser line. Viable cells with Hoechst-positive and PI-negative nuclei, and dead cells with Hoechst-positive and PI-positive nuclei, were counted in 2 or 3 images of each tissue slice per group. Data were obtained from 3 individual experiments. One-way ANOVA was used, and $P < .05$ was considered to be statistically significant.

Irradiation of Glioblastoma Slice Cultures

Photon irradiation of slices was performed at the Department for Radiation Therapy and Radio-oncology, University of Leipzig, with a 150-kV X-ray unit (DARPAC 150-MC) with an energy of 13.2 mA and a dose rate of 0.86 Gy/min. Cell culture plates were placed under a specially constructed plate device and irradiated until the desired dose was reached. Alternatively, photon irradiation was performed using the GSI X-ray device (GE Isovolt Titan 320, 250 kV, 16 mA) at a dose rate of 1.4 Gy/min.

HI irradiation with a carbon beam was performed at GSI (Gesellschaft für Schwerionenforschung), Darmstadt, at the former patient irradiation site. The ion beam was generated at the SIS18 synchrotron facility and delivered in a spread-out Bragg peak (SOBP[33]) as used in carbon ion therapy. The dose applied to the slices was 2 or 4 Gy in a 50-mm-width SOBP corresponding to a linear energy transfer range of 50–70 keV/μm. With this method, the target tissue volume is distributed into voxels in a treatment plan. Then, the ion beam is directed at the 3-dimensional tumor volume, using active energy variation and the raster scanning technique. For experiments with slice cultures, the volume was defined as the area and the height of 1 well. Before and after irradiation, slice cultures were kept in an incubator as previously described here and were removed for only about 15 min for transport and to place them on the irradiation belt.

After irradiation, slices were fixed in 4% paraformaldehyde after one of several time points, washed in PBS, and further processed for paraffin embedding or cryosectioning. Cryosections were cut at 14 μm and stored at −80°C until further use. Paraffin sections were prepared at 8 μm, dried, and stored at room temperature.

For staining of DNA DSBs, cryosections were dried for 20 min at room temperature and then washed twice in PBS and incubated with 1.5% Triton/PBS for 10 min. Then sections were blocked with 10% normal goat serum in 1.5% Triton/PBS for at least 1 h, followed

by incubation overnight at 4°C with γH2AX primary antibody (mouse monoclonal, 1:100; Millipore). Then, sections were washed 3 times with PBS and incubated with the secondary antibody (goat anti-mouse 1:1000; Alexa 488, Invitrogen) for 1 h, washed again, counterstained with Hoechst 33342, and mounted with Dako fluorescent mounting medium. Z-stacks were taken using a Zeiss LSM 510 confocal microscope at 400× magnification at intervals of 2 μm. Paraffin sections were stained as previously described here.

Results

Slice Cultures From Glioblastoma: Histology and Survival

Slices were at first cut with a vibratome and survived well, with histological preservation of the main features of the original tumor for at least 16 days (Fig. 1). At later stages, cell density appeared to decline in some tumor slices, whereas cells in other slices survived longer (Fig. 1E–H and I–L). Some tumors, however, were difficult or even impossible to cut due to their viscous texture, which may have resulted from altered collagen expression.[18,34] Using a tissue chopper resolved this problem with equally good histological preservation and maximal survival time. Histological examination of cultured GBM slices and comparison with the original neuropathology used for diagnosis confirmed striking maintenance of the general and individual hallmarks of the tumor (pleomorphic nuclei, palisading, invading vessels/neovascularization, and necrotic areas). As expected, GBM stained positive for GFAP, nestin (intermediate filament protein, GBM "stem" cell marker), and vimentin (mesenchymal intermediate filament protein) and differed strongly in their cell density and content of necrotic areas (Fig. 2).

Irradiation of Slices: Effect on Proliferation

Ionizing radiation is known to cause DNA DSBs. If DNA repair cannot be conducted properly, cell proliferation is arrested at a cell cycle checkpoint and either is inactivated (eg, in postmitotic cells) or proceeds into programmed cell death. Here, we tested the dose- and time-dependent decrease of the proliferation index after SOBP carbon or X-irradiation. GBM slices were fixed 6 or 24 h after irradiation and processed for paraffin sectioning. Proliferating cells were labeled with a Ki67 antibody (Fig. 3A–D), which marks cells in every state of the cell cycle except the G_0 resting phase.[35] The percentage of Ki67-positive cells in relation to the total number of nuclei was calculated to express the proliferation index. Carbon ions did not significantly diminish proliferation at 6 h, but after 24 h a reduction of ~40% was found ($P = .018$; Fig. 3E). This is the first demonstration of specific effects of HI on human GBM tissue ex vivo. Photons also showed the anticipated time-dependent reduction of proliferation (here, ~50% after 24 h; Fig. 3F). Thus, GBM-derived slice cultures can be irradiated and the biological effects of photons and HI on tumor cells and mechanisms of resistance can be studied in this model.

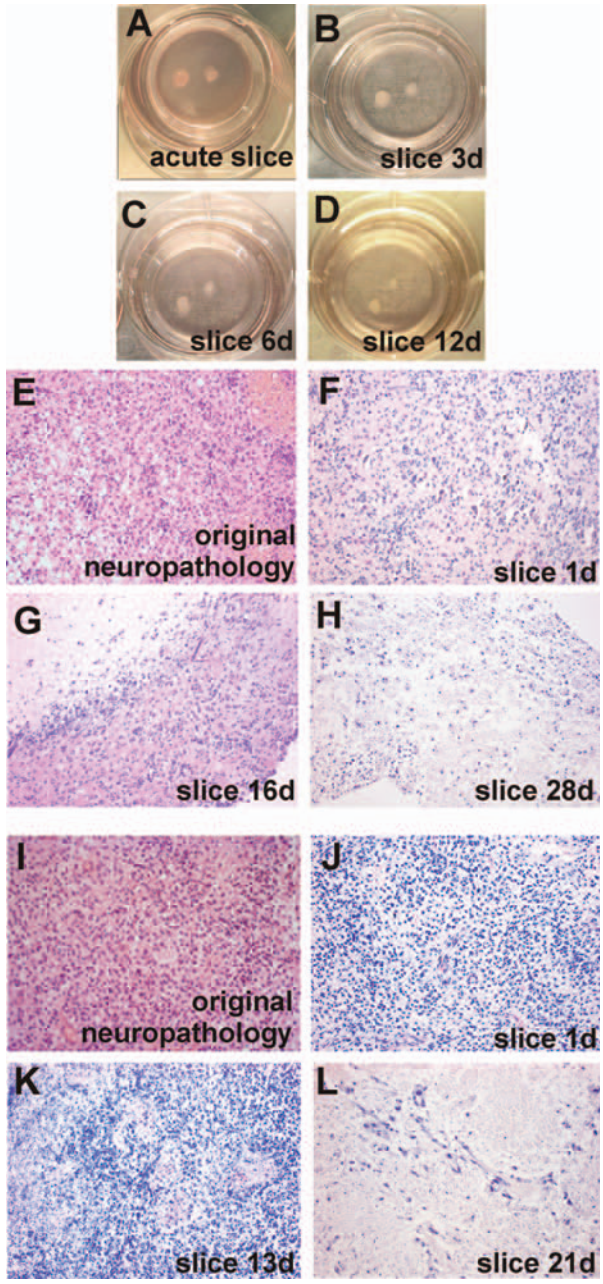

Fig. 1. Human GBM slices in culture. Slices were cultured on membrane inserts in six-well plates with no signs of degeneration in acute (A) slices at 1 day or at 3 days (B), 6 days (C), or 12 days (D) in vitro. Original H&E neuropathology (E and I) and H&E-stained paraffin-embedded sections (8 μm; F–H and J–L) prepared from slices after various culture periods. Two different tumors (E–H and I–L) are shown. Note that typical features of individual tumors were maintained at least from 1 to 16 days (F–G) and 1 to 13 days (J–K) in vitro; massive cell loss was observed after 20 days in vitro (H and L). Original magnification: 1× in A–D; 200× in E–L.

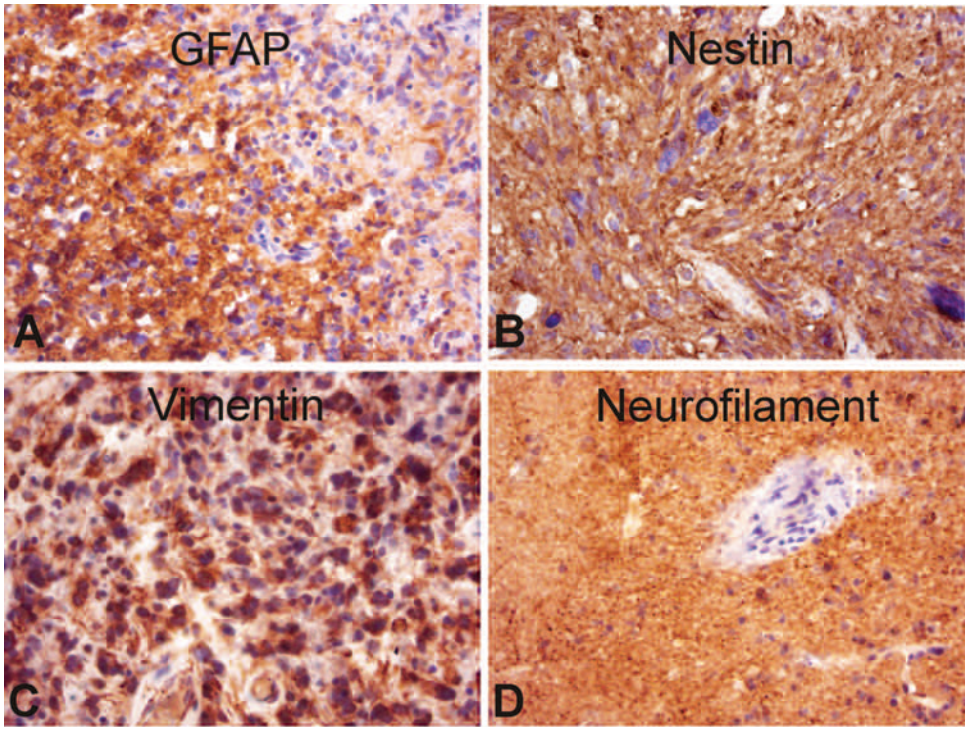

Fig. 2. Typical GBM markers expressed in human GBM slice cultures. Slices were fixed after 7 days in culture and processed for paraffin sectioning (8 μm). Characteristic marker proteins were visualized by antibody staining for GFAP (A), nestin (B), vimentin (C), and neurofilament (D). Counterstaining was performed with hematoxylin. Original magnification: 400×.

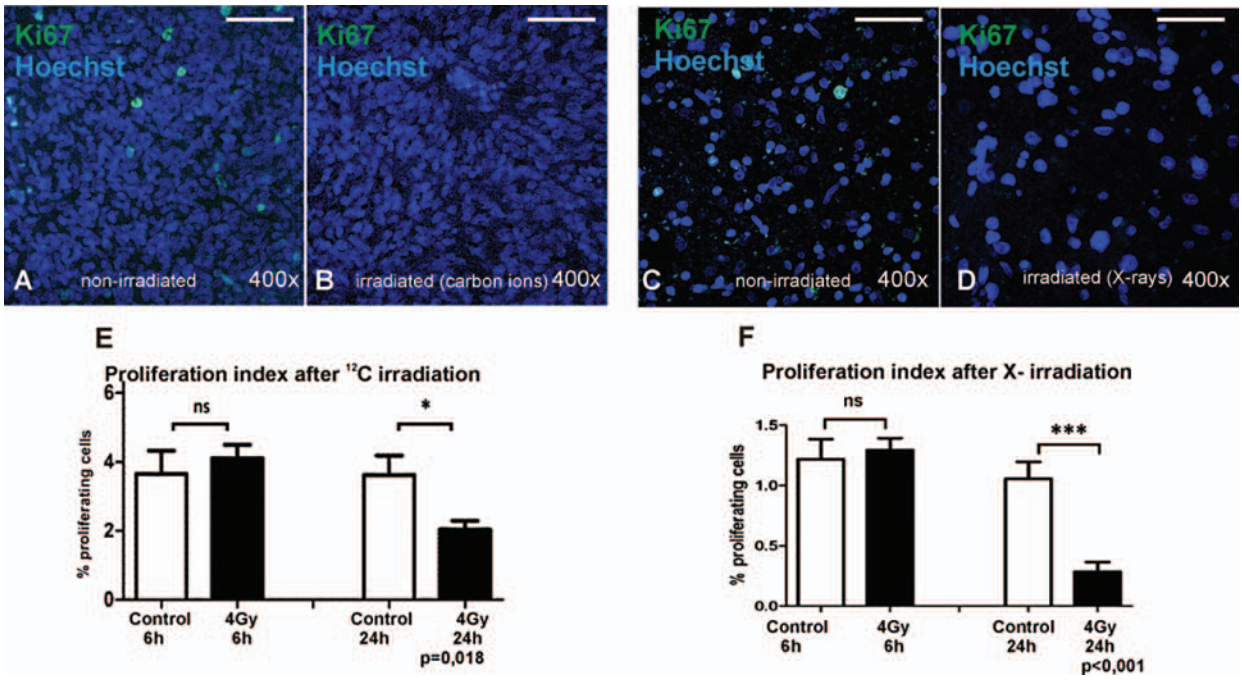

Fig. 3. Proliferation index of human GBM slice cultures irradiated with X-rays or carbon ions. Slices were treated with either 4 Gy of SOBP carbon ions at GSI or 4 Gy of X-rays and fixed 6 or 24 h later. Proliferating cells were then visualized in paraffin-embedded sections (8 μm) using a Ki67 antibody (green) combined with nuclear counterstaining (Hoechst 33342; blue) for quantitative analysis. (A), Non-irradiated; (B), irradiated with 4 Gy carbon ions; (C), non-irradiated; (D), irradiated with 4 Gy X-rays; scale bar = 50 μm). The proliferative fraction in relation to total cell number was determined in at least 12 pictures per group by using ImageJ software. After 6 h, the effect on proliferation was not yet significant after both irradiation types, but after 24 h both treatments resulted in a significant decrease of Ki67-positive cells (E and F).

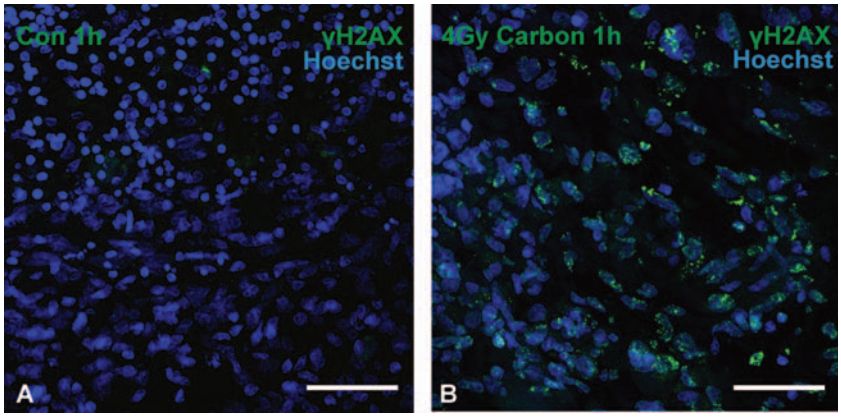

Fig. 4. DNA damage in GBM slices after carbon irradiation. Slices were irradiated with 4 Gy of carbon ions in an SOBP and fixed 1 h later. Paraffin sections (8 μm) were assembled and DNA DSBs were visualized by immunocytochemistry with a γH2AX antibody (green) and nuclei with Hoechst (blue). Although γH2AX was rarely detected in non-irradiated controls (A), exposure to therapeutic heavy ions caused massive induction of phosphorylation of H2AX (B). Scale bar = 50 μm. Original magnification: 400×; confocal Z-stacks.

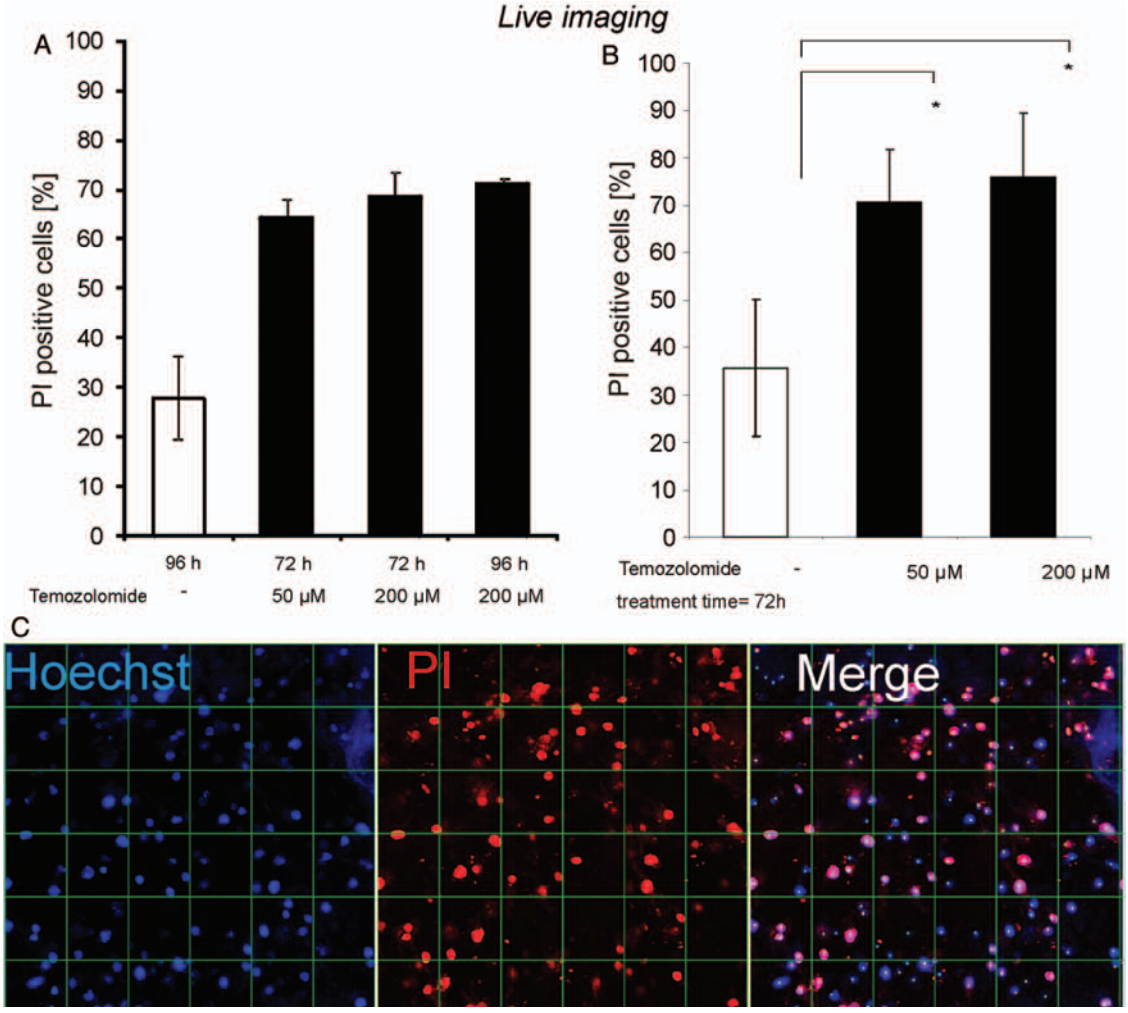

Fig. 5. Live imaging of cell death in GBM slice cultures treated with TMZ. Slices were treated with either 50 or 200 μM TMZ and then incubated for 72 or 96 h (A). After determination that an incubation of 72 h was sufficient, the experiment was repeated 3 times with 50 or 200 μM TMZ (B). Dying cells were labeled with PI (red), and all nuclei were counterstained with Hoechst 33342 (blue) for quantitative analysis (C). Grid distance = 50 μm. Confocal images were taken and nuclei digitally counted. Student's *t*-test was performed, and $P < .05$ was considered statistically significant. Both concentrations showed a significant increase in dying cells compared with controls, with the effect slightly more pronounced at 200 μM. Original magnification: 200× in C; confocal Z-stacks.

Exposure of Slices to Carbon Ions and Detection of DNA Damage

After induction of DNA DSBs by ionizing radiation, repair proteins are rapidly recruited to the sites of damage. In mammalian cells, this involves a cascade of proteins that ultimately allows repair of the breaks in the form of rejoining the loose DNA ends.[36,37–39] When we established the irradiation setup of GBM slice cultures, we wanted to test whether the slices were evenly hit by the beam, and therefore we used γH2AX as an early DSB marker for the visualization of DNA damage. γH2AX describes a phosphorylation of histone 2AX at serine 139 around the region of the DSB,[40] which allows binding of further repair proteins, such as MDC1 and 53BP1. Once the repair process is completed, the repair proteins dissociate and H2AX is dephosphorylated by a distinct phosphatase complex.[41] Slices were irradiated with 4 Gy carbon ions in an SOBP to match therapeutic conditions, fixed after 1 h, and embedded in paraffin, and sections were stained with an antibody to γH2AX. γH2AX was mostly absent in controls but appeared in a punctuated nuclear pattern in all sections, confirming induction of DNA damage throughout the irradiated tissues (Fig. 4).

Exposure of Glioblastoma Tissue Slices to Temozolomide: Detection of Cell Death Using Live Imaging

TMZ is the current gold standard in chemotherapy of GBM.[42–46] It is an alkylating agent that, in an aqueous solution at physiological pH, dissolves into its bioactive form MTIC (5-(3-methyl-1-triazeno)imidazole-4-carboxamide), which is capable of penetrating the blood–brain barrier.[47–49] To test whether GBM tissue in culture responds to TMZ treatment, slices were incubated with vehicle control or TMZ (50 or 200 μM). After 72 or 96 h, Hoechst 33342 and PI were

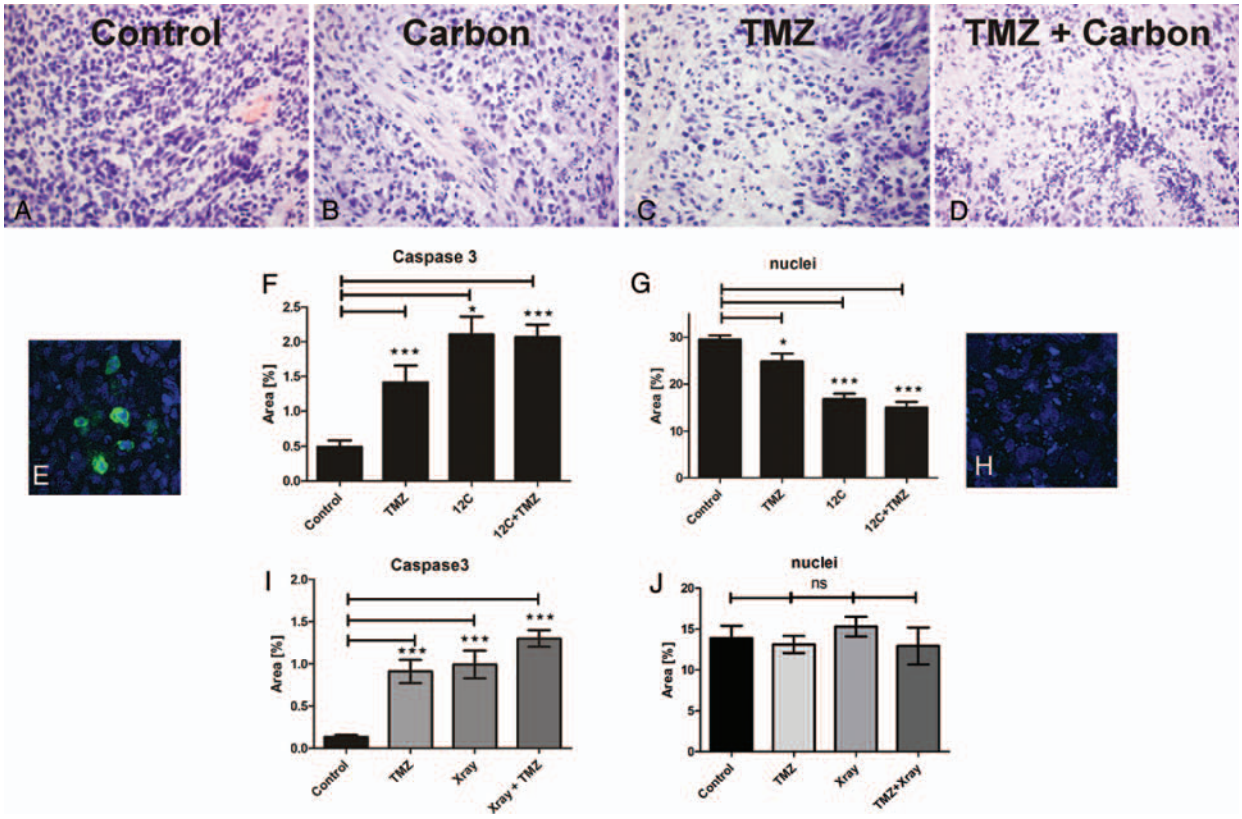

Fig. 6. Combined treatment of TMZ and irradiation with carbon ions and X-rays on human GBM slice cultures. GBM slices were treated with TMZ (200 μM), X-ray irradiation (4 Gy), SOBP carbon ions (2 Gy), or irradiation + TMZ. TMZ treatment started 24 h before irradiation and was maintained throughout the entire incubation time. Slices were fixed 2 days after irradiation, and H&E staining was performed for neuropathological assessment (A–D). In addition, activated caspase 3 was labeled (green in E), and fragmented nuclei were visualized using Hoechst 33342 (blue in E and H). Pictures of immunofluorescent stainings (in RGB format) were split into the three single channels of red, green, and blue, resulting in 3 gray-value pictures. The analysis was then performed using ImageJ's Area Measurement function. The area coverage is represented by white pixels in the respective channels and can be fine-tuned by threshold adjusting. In the green channel, the positive area represents the caspase 3–positive cell population, and in the blue channel, the area covered by nuclei is displayed (E–J). Carbon significantly reduced cell numbers (G), whereas X-rays did not (J). Significance was determined using GraphPad Prism 5 (1-way ANOVA, Bonferroni test). Both treatments result in a significant increase in cell death and morphological alterations compared with the vehicle-treated control. Original magnification: 400× in A–F.

added to the cultures to visualize intact and dying nuclei using confocal live imaging. Microimages were taken in which dying cells (Hoechst/PI double labeled) were counted and their number related to the total number of nuclei (Hoechst single labeled), allowing calculation of a cell death rate. TMZ-induced cell death was highly significant at 72 h, with little or no further increase until 96 h. This effect was more pronounced in slices treated with 200 μM compared with 50 μM of TMZ (Fig. 5). Thus, chemotherapeutic effects can be mimicked in GBM slices and observed over time in situ.

Exposure of Slices to Carbon Ions and TMZ: Detection of Cell Death Using Activated Caspase 3 Staining

Slices were exposed to either TMZ or carbon ions in an SOBP alone or in combination and fixed 48 h after irradiation. TMZ treatment was started 24 h before irradiation, and a second dose was applied with the regular change of medium 48 h later. At the end of the treatment phase, induction of programmed cell death was determined using cleaved caspase 3 and H&E staining. All treatment regimens caused a decrease in cell density and nuclear alterations (Fig. 6B–D). In some slices, intact nuclei could no longer be identified because only fragments remained. Therefore, instead of relating damaged cells to a total number of cell nuclei, the area coverage of caspase 3–positive (green) and Hoechst-positive (blue) pixels (Fig. 6E, caspase 3–positive cells; 6H, nuclear fragments) was calculated as a measure of treatment-induced damage. After all treatments, the area of caspase 3–positive pixels was significantly increased, whereas Hoechst-positive pixels were decreased (Fig. 6F and G). Both irradiation and TMZ alone induced

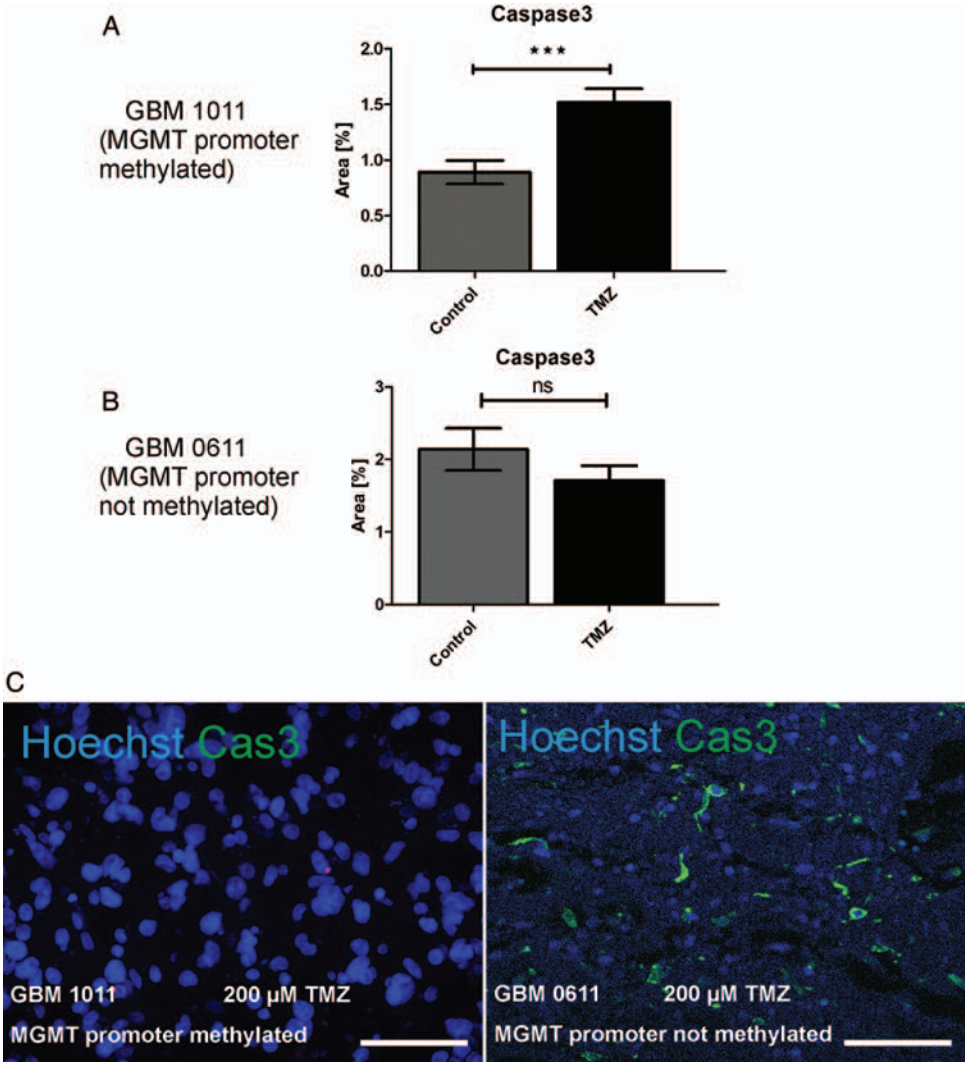

Fig. 7. Induction of cell death. GBM slice cultures with different MGMT promoter methylation states were tested for their response to TMZ treatment. In a GBM specimen with methylated promoter sequence (A), TMZ treatment resulted in significant induction of caspase 3 cleavage (GBM 1011), whereas caspase 3 activation was not significantly induced (B) in GBM with an unmethylated promoter sequence (GBM 0611). Immunocytochemistry is shown for the methylated (C, right) and unmethylated (C, left) tumor for cleaved caspase 3 (green) and nuclei (Hoechst, blue). Scale bar = 50 μm. Original magnification: 400× in C; confocal Z-stacks.

cell death, but a combination of both treatments did not show a synergistic or additive effect in the cases studied. Irradiation with X-rays, however, also activated caspase 3 but did not cause significant cell loss (Fig. 6I and J). Thus, effects of established treatment options on cell death can be studied in GBM slices.

TMZ Treatment of Slice Cultures From Tumors With Different MGMT Promoter Methylation Statuses

Lack of promoter methylation of O^6-methylguanine-DNA methyltransferase (MGMT) has been reported to significantly decrease patients' susceptibility to TMZ and thus survival,[50–52] but there are also patients with nonmethylated promoter who benefit from TMZ treatment. In fact, we identified one tumor in which TMZ did not significantly induce cell death and we therefore requested the promoter methylation status, which is assessed by quantitative PCR and sequencing techniques of tumor material obtained from surgery.[53–55] This tumor indeed turned out to have a nonmethylated MGMT promoter (Fig. 7). However, in line with the clinical observations that some patients with nonmethylated MGMT respond to TMZ, we subsequently identified other tumors in which TMZ significantly enhanced activation of caspase 3 to levels comparable to those of the methylated tumor (Fig. 8).

Discussion

Organotypic slice cultures derived from early postnatal rodent brain[56] are widely used in neuroscience due to their easy access for pharmacological intervention, electrophysiological studies, and live imaging. We have employed entorhinohippocampal preparations, which, due to the orientation of the trisynaptic pathway (perpendicular to the longitudinal axis of the hippocampus), allow for maintenance of the major connectivity.[12,16] In this study, we adjusted cutting and culturing methods to prepare slices from human GBM and demonstrated evidence for their suitability as a test system for novel therapies including irradiation with HI. We irradiated GBM slices with photons and carbon ions and in both instances found that radiation induced DNA damage and strongly affected proliferation. Carbon ion radiation also induced activation of caspase 3, a potent inductor of programmed cell death (Figs. 3–6). In contrast to photon radiation, which delivers energy all the way through the body (eg, in an anterior-posterior direction), the depth of energy deposition of HI can be adjusted and limited to distinct areas of a few millimeters.[57,58] In fact, much hope derives from successful intensity-modulated carbon therapy of chondrosarcomas of the skull base, a treatment first established at GSI.[59–61] An accelerator specialized for medical applications has been constructed in Heidelberg and opened for patients in 2009. Currently, clinical trials and work with cell lines are aimed at testing the effects of carbon ion radiation in tumors other than chondrosarcomas. Our data support observations in GBM-derived cell lines[62,63] and in a small group of patients,[64,65] and our approach may lead to a more detailed understanding of the biological effects of HI and additional novel therapies. Moreover, surviving tumor cells can be studied to understand their mode of resistance.

We also applied TMZ to GBM slices and analyzed the effect on cell survival using PI staining and live imaging, as well as labeling of activated caspase 3 in paraffin-embedded sections. TMZ is an alkylating agent widely used to treat GBM that, in combination with radiation therapy, helps prolong patients' survival time. Survival depends on the methylation status of the promoter of the repair enzyme MGMT. Methylation was significantly more frequent in patients who survived longer than 36 months after surgery ($P < .05$).[66] Statistically, methylation status apparently affects susceptibility to TMZ,[50,67] but some patients with nonmethylated status also seem to benefit from TMZ. Of note, determining methylation status is not trivial, as only a few of the 109 potential sites have been tested.[68] A recent survey among 1053 members of the neuro-oncology community in the United States found that only a small percentage (10.9%) of clinicians regard MGMT status as "always" or "almost always" helpful for their decision making.[69] In line with this observation, we identified 2 nonmethylated tumors that were resistant to TMZ (Fig. 7) but also found others that were not

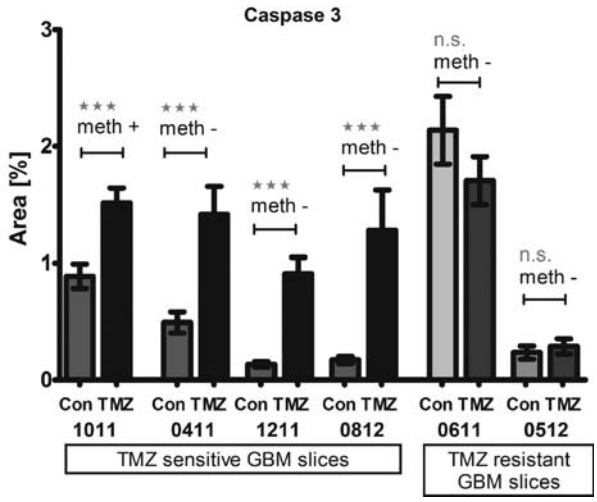

Fig. 8. Caspase 3 activation in GBM slices after TMZ treatment independent of MGMT promoter methylation. After TMZ treatment, caspase 3 activation was detected in some specimens, whereas others seemed to be resistant. This was independent of MGMT promoter methylation. From left to right: GBM 1011 with methylated MGMT promoter (meth +) and significant caspase 3 activation; GBM 0411 with nonmethylated MGMT promoter (meth -) and significant caspase 3 activation; GBM 1211 with nonmethylated MGMT promoter (meth -) and significant caspase 3 activation; GBM 0812 with nonmethylated MGMT promoter (meth -) and significant caspase 3 activation; GBM 0611 with nonmethylated MGMT promoter (meth -) and nonsignificant (ns) caspase 3 activation; GBM 0512 with nonmethylated MGMT promoter (meth -) and nonsignificant (ns) caspase 3 activation. (*t*-Test, $P < .05$)

(Fig. 8). Thus, the next challenge will be to relate TMZ-induced cell death rates obtained in slices to (progression-free) survival times; we will address this issue with tumor-derived samples during the next 2 years to test the predictive value of this assay.

It may be trivial to state that only in vitro systems allow different therapeutic options to be tested for an individual patient. Our data demonstrate that tumor-derived GBM-slice cultures in principle are suitable for that task as a step on the way to more personalized therapies while also helping unravel basic mechanisms of tumor resistance.

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Conflict of interest statement. None declared.

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5. Zusammenfassung

Dissertation zur Erlangung des akademischen Grades
Dr. rer. med.

Titel: Organotypische Slicekulturen von humanem *Glioblastoma multiforme* als Testsystem für neue Therapien

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Glioblastoma multiforme (GBM) ist ein häufig vorkommender, aggressiver Hirntumor, der unbehandelt binnen weniger Monate zum Tode führt. Die heutige Standardtherapie, eine Resektion des größtmöglichen Tumorvolumens mit anschließender Kombination aus Chemo- und Radiotherapie mit Temozolomid und Röntgenstrahlung, resultiert in einem mittleren Überleben von Patienten von ca. 15 Monaten. Der initiale Tumor wird zwar entfernt, jedoch treten meist Rezidive auf, die durch invasive GBM-Zellen oder nicht vollständig abgetötete Tumorstammzellen (*cancer stem cells*, CSC) verursacht werden. In der Forschung wird seit Jahrzehnten intensiv versucht, die Pathogenese von GBM aufzuklären und somit wirksamere Therapieoptionen zu finden. Dies wird durch die Heterogenität von GBM an sich, verursacht durch eine Vielzahl möglicher unterschiedlicher Mutationen von Rezeptoren, Promotoren oder Molekülen in Signalkaskaden, aber auch das individuell unterschiedliche Ansprechen von Patienten auf Therapien, erschwert.
Oft wird Grundlagen- oder präklinische Forschung an GBM-Zelllinien, Primärkulturen aus Biopsien oder Tiermodellen durchgeführt. Dies ist mit Schwierigkeiten verbunden, da sich die Ergebnisse meist nicht in der Klinik bestätigen lassen. Die Ursache dafür liegt in den Nachteilen der einzelnen verwendeten Modelle begründet. So reagieren Zellen in Kultur anders als in einem Organismus, da ihnen der 3D-Gewebeverband, die extrazelluläre Matrix fehlt. Über lange Zeit subkultivierte Zelllinien sind auch oft soweit mutiert bzw. auf bestimmte Subtypen von Zellen selektiert, dass sie mit der ursprünglichen Zellprobe kaum noch Gemeinsamkeiten haben. In Zellkulturen und auch in Tiermodellen mit immunsupprimierten

Nagern fehlt zudem die immunologische Komponente, die es problematisch macht, Ergebnisse auf Patienten zu übertragen.
In dieser Arbeit wurde ein Modell etabliert, das es ermöglicht, humanes GBM-Gewebe aus Resektionen über bis zu vier Wochen in Kultur zu halten. Dazu wird das Gewebe in scheibenförmige Gewebeschnitte mit einer Dicke von 350 µm zerteilt und auf speziellen Membranen an einer Grenzfläche zwischen Kulturmedium und Luft kultiviert. Die so gewonnenen Schnittkulturen (Slices) beinhalten alle GBM-typischen Zellen sowie Endothel und die extrazelluläre Matrix, was zu einer möglichst nativen Gewebestruktur beiträgt. Die morphologische Charakteristik des individuellen Tumors bleibt auch nach längerer Kulturzeit erhalten. Es werden GBM-typische Markerproteine wie GFAP oder Nestin exprimiert, und Proliferation findet bis zu zwei Wochen in Kultur auf einem relativ gleichbleibenden Niveau statt. Nach dieser Zeit zeigt sich eine erhöhte Zelltodrate, die in einer geringer werdenden Zelldichte in den Schnittkulturen resultiert.
Für diese GBM-Schnittkulturen wurde ein Setup zur Bestrahlung mit konventioneller Röntgen- bzw. Photonenstrahlung und auch mit neuartiger Kohlenstoff-Ionenstrahlung etabliert. Beide Strahlenarten führten in ersten Experimenten zu DNA-Schäden in Form von Doppelstrangbrüchen (DSBs), einem Rückgang der Proliferation sowie erhöhter Apoptoserate im Vergleich zu unbestrahlten Kontroll-Schnitten. Auch das klinisch relevante Zytostatikum Temozolomid (TMZ) wurde auf GBM-Schnittkulturen appliziert und mit Photonen- und Kohlenstoff-Ionenstrahlung kombiniert. Hier zeigten sich patientenspezifische Unterschiede im Ansprechen auf die Behandlung. Dies war nicht unbedingt abhängig von einer Methylierung der Promotorsequenz für ein Reparaturenzym (O^6-Methylguanin-DNA-Methyltransferase, MGMT). Der Verlust der Promotor-Methylierung wird klinisch mit einer erhöhten Resistenz gegen TMZ-Behandlung assoziiert, allerdings gibt es auch Hinweise auf Ausnahmen. Somit reflektiert die variierende Reaktion der GBM-Schnittkulturen Beobachtungen aus der Klinik und macht das Modell zu einem interessanten humanen Testsystem zur Aufklärung von Resistenzmechanismen gegenüber etablierten Therapien sowie für neue Wirkstoffe bzw. chemo- radiotherapeutischer Behandlungsregime. Auch könnte es zukünftig für eine Optimierung einer Therapie individueller Patienten genutzt werden, da aus dem Gewebe einer Resektion mehrere Schnittkulturen angelegt und somit mehrere Wirkstoffe bzw. Kombinationen untersucht werden können.

6. Summary

Glioblastoma multiforme (GBM) is an aggressive brain tumor which is lethal within several months if untreated. Today, the standard therapy consists of resection of a maximum tumor volume followed by combined radio- chemotherapy with X-irradiation and temozolomide. With this therapy, the average patient survival could be increased to approximately 15 months but it cannot avoid a relapse due to infiltrating tumor cells or resistant cancer stem cells (CSC). Extensive research has been performed on the mechanisms of GBM pathogenesis in order to find more successful therapy regimes. There has been no major breakthrough during the last decades, which could be due to the great heterogeneity of GBM caused by various mutations of receptors, promoters or signaling molecules, but also the individual patient responses to therapies.

Basic research or preclinical studies often employ GBM-cell lines, primary cultures derived from biopsies or animal models. The results are difficult to translate into a human setting relevant for clinical use because these models contain some major drawbacks. Cells in culture react differently than those in an organism because they lack their natural 3D-environment, the extracellular matrix. Cell lines also tend to mutate after a series of passages, or a more robust subtype replaces sensitive or less proliferative cells resulting in a culture which does not resemble the original biopsy anymore. Cell cultures as well as animal models with immunosuppressed rodents are devoid of an immune response which makes it difficult to translate results from these models into a clinical setting.

In this work, we established a human GBM-model from resections which allows for cultivating GBM tissue for up to four weeks. The tissue is dissected into 350 µm thick slices and cultivated on membranes on a liquid-air-interface. These 3D-slice cultures include all initial cell types as well as endothelium and the extracellular matrix which results in a setting resembling the tumor *in vivo*. The individual tumor characteristics are preserved over a longer culturing period. The typical GBM marker proteins GFAP and Nestin are expressed and proliferation stays on a relatively constant level for up to two weeks. After that, a higher cell death rate can be observed which results in a decreased cell density in slice cultures.

We established a setup to irradiate human GBM slice cultures with conventional X-rays or photons as well as the heavy ion carbon, which is a new radiotherapy option. Both radiation types yielded DNA double strand breaks (dsbs), a decrease in proliferation and an increase in apoptosis induction compared to untreated control slices. We also applied the cytostatic compound temozolomide, which is widely used in GBM therapy, alone or in combination with photon or carbon irradiation. The outcome of these experiments was different for GBM tissue from individual patients, but independent from MGMT promoter methylation. This parameter is associated with clinical outcome and a worse prognosis if the promoter sequence of the

DNA repair enzyme O^6-methylguanine-DNA- methyltransferase (MGMT) is de-methylated. Loss of promoter methylation results in a higher activity of the enzyme and is thought to be connected to TMZ resistance. In a clinical setting, this parameter is not reliably predictive because there are many patients whose responses to TMZ therapy do not correlate with MGMT promoter methylation. To this end, the human GBM slice culture model reflects the clinical situation and could be suitable as a human test system to detect mechanisms of resistance against known or new compounds and help finding novel radio-chemotherapeutic treatment regimes. In the future, it could be used to optimize therapy for individuals by testing several different treatments on one slice culture from resected material of the respective patient.

7. Erklärung über die eigenständige Abfassung der Arbeit

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig und ohne unzulässige Hilfe oder Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Ich versichere, dass Dritte von mir weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen, und dass die vorgelegte Arbeit weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde zum Zweck einer Promotion oder eines anderen Prüfungsverfahrens vorgelegt wurde. Alles aus anderen Quellen und von anderen Personen übernommene Material, das in der Arbeit verwendet wurde oder auf das direkt Bezug genommen wird, wurde als solches kenntlich gemacht. Insbesondere wurden alle Personen genannt, die direkt an der Entstehung der vorliegenden Arbeit beteiligt waren.

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